Creation of a Humanized Mouse Model to Study Islet Graft Rejection

by

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ABSTRACT

Background

Intraportal islet transplantation is a suitable method to treat type 1 diabetes. However, this treatment option is limited to a small subpopulation of patients with diabetes. For islet transplantation to be a more sustainable treatment option, major obstacles must be overcome. An alternative supply of islets must replace the unsustainable human islet source and an alternative method must be developed reduce the usage of systemic immunosuppressive medications. Before alternative islet sources and novel immunosuppressive strategies can be used in clinical practice, it is important to conduct immunological studies using a variety of islet sources in a reliable model that realistically mimics the human immune system.

To achieve this, an appropriate humanized mouse model must be developed, and quantitative methods must support the rejection of a variety of islet sources. In addition, it is important for qualitative observations to further reinforce the quantitative analytical observations. The main objectives of this thesis are twofold: 1) to use NSG-MHC I/II double knockout (NSG) mice to support the survival of human (HI), neonatal porcine (NPI), and stem cell-derived islets (SC-β), and 2) to display islet graft rejection in these mice via a reversal to the hyperglycemic state, or through functional rejection of stimulated insulin secretion, after reconstitution with human peripheral blood mononuclear cells (PBMCs) without graft-vs-host disease (GVHD).

Methods

A brief investigation was conducted to assess the native immune system in mice using Immunofluorescence (IF). A random selection of HI transplanted mice was stained using anti-

mouse CD4, CD8, and CD68 T-cells and human insulin. Metabolic follow-up in streptozotocin-induced diabetic mice transplanted with HI and SC-βs was determined by examining blood glucose values pre- and post-reconstitution, and a reversal of blood glucose to hyperglycemic levels indicated islet graft rejection. Metabolic follow-up in naïve, non-diabetic mice transplanted with NPIs was determined by comparing a 4-week post-transplantation intraperitoneal glucose tolerance test (IPGTT) and at 2-, 4-, 5-, and 6-weeks post-reconstitution IPGTT and stimulated serum porcine insulin was obtained and analyzed. Stimulated porcine insulin values at or near the lowest limit of detection on the enzyme-linked immunosorbent assay (ELISA) curve for both basal (time 0) and stimulated (time 60) levels were considered as complete porcine islet graft rejection.

To determine the possibility of GVHD in these mice, the weight values of naïve, non-diabetic NPI transplanted mice were taken three times weekly and compared to baseline values. Loss of more than 20% of initial weight indicated the presence of GVHD.

To demonstrate the ability of SC-β transplanted mice to secrete insulin, an IPGTT was conducted at 8- and 12-weeks post-transplantation prior to reconstitution, and stimulated insulin values were obtained. Further exploration of the cell composition of SC-βs and outcome of transplantation were explored in Appendix A.

Immunohistochemistry (IHC) and IF imaging was used to determine the presence of human immune cell infiltration in the graft region as well as co-localization of human immune cells in relation to insulin or chromogranin A using mice in all three groups (HI, NPI, SC-β).

Results

Investigation of the native immune system displays an absence of mouse CD4$^+$ and CD8$^+$ T-cells. CD68$^+$ T-cells were identified, though are stated to be functionally defective in these mice. Prior to reconstitution, mice transplanted with HI and SC-β demonstrated graft functionality through normalized blood glucose values. Post-reconstitution, HI and SC-β transplanted mice displayed a reversal to hyperglycemia between 5 to 18 days and 21 to 23 days, respectively. IHC and IF imaging confirmed the infiltration and co-localization of human immune cells in relation to insulin positive cells in the kidney graft. 53.8% of NPI transplanted mice displayed complete islet graft rejection at 6-weeks post-reconstitution. Mice did not decrease their weights by more than 20% from baseline values indicating the absence of this GVHD symptom. Co-localization studies confirmed the infiltration and human immune cells in relation to chromogranin A positive cells.

Conclusions

These results display the feasibility of transplanting a wide variety of islet sources in NGS mice. Results demonstrate that reconstitution of mice with peripheral blood mononuclear cells exhibits islet graft rejection and that this mouse model can be adapted for future strategies, including the use of hypoimmunogenic stem cells and encapsulation strategies.

PREFACE

Some of the research conducted for this thesis forms part of a domestic research collaboration, led by Professor Cristina Nostro, with Professor Gregory Korbutt and Professor Andrew Pepper being the collaborators at the University of Alberta. The differentiation of stem cell-derived islets was performed by Farida Sarangi, Angel Singh, and Dr. Cristina Nostro. The data analysis in chapter 2 and appendix A are my original work, as well as the literature review in chapter 1.

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α – Alpha

β – Beta

δ – Delta

ε – Epsilon

γ – Gamma

GLOSSERY OF TERMS

α-Gal – galactosyl alpha (1-3) galactose

ABC – Avidin-biotin complex

ACE – Anterior chamber of the eye

APC – Antigen-presenting cell

API – Adult porcine islets

ATP – Adenosine triphosphate

AUC – Area under the curve

BAT – Brown Adipose Tissue

Bcl-2 – B-cell lymphocyte 2

BG – Blood glucose

BMI – Body mass index

cAMP – Cyclic adenosine monophosphate

CD – Cluster of differentiation

CDKN1A – Cyclin dependent kinase inhibitor 1A

CgA – Chromogranin A

CNI – Calcineurin Inhibitor

CNS – Central Nervous System

CRH – Corticotropin releasing hormone

CRISPR/Cas9 – Clustered regularly interspaced short palindromic repeats associated protein 9

CsA – Cyclosporine A

CTLA4-Ig – Cytotoxic T-lymphocyte-associated protein 4 immunoglobulin

CVB – Coxsackievirus B

DAB – 3,3-diaminobenzidine

DC – Dendritic cells

DCCT – Diabetes control and complications trial

DKO – Double knockout

DM – Diabetes Mellitus

DNA – Deoxyribonucleic acid

DSA – Double strand break

ELISA – Enzyme-linked immunosorbent assay

FFA – Free fatty acids

FICC – Fetal porcine islet-like cell cluster

FPP – Fetal pig pancreas

FTO – Fat mass and obesity-associated

GAD – glutamic acid decarboxylase

GDM – Gestational diabetes mellitus

GH – Growth hormone

GIP – Glucose-dependent insulinotropic polypeptide

GLK – Glucokinase

GLP-1 – Glucagon-like peptide-1

GLP-1R – Glucagon-like peptide-1 receptor

GLUT – Glucose transporter

GSIS – Glucose stimulated insulin secretion

gRNA – Guide ribonucleic acid

GVHD – Graft versus host disease

GWAS – Genome wide association study

HbA1C – Glycated hemoglobin

hESC – Human embryonic stem cells

HHS – Hyperosmolar hyperglycemic state

hiPSC – Human-induced pluripotent stem cells

HIV – Human immunodeficiency virus

HLA – Human leukocyte antigen

HNF – Hepatocyte Nuclear Factor

hPL – Placental lactogen

hPSC – Human pluripotent stem cells

HSC – Hematopoietic stem cells

IAA – Insulin autoantibody

IA-2 – Islet antigen-2

IAPP – Islet-amyloid polypeptide

IBMIR – Instant blood mediated inflammatory reaction

IEQ – Islet Equivalents

IFN – Interferon

IL – Interleukin

IL-R – Interleukin receptor

IL2rg – Interleukin 2 receptor common gamma chain

INS – Insulin gene

INT – Intensive therapy group

IP – Intraperitoneal

IPC – Insulin producing cells

IPGTT – Intraperitoneal glucose tolerance test

IPS – Induced pluripotent cell line

IRS-1 – Insulin Receptor Substrate-1

IV – Intravenous

KC – Kidney capsule

KCNJ11-Kir6.2 – Potassium inwardly rectifying channel subfamily J member 11

KCNQ1 – Potassium voltage-gated channel subfamily Q member 1

kg – Kilogram

lbs – Pound

MAFA – Musculoaponeurotic fibrosarcoma oncogene family A

MHC – Major histocompatibility complex

MODY – Maturity onset diabetes of the young

mRNA – Messenger ribonucleic acid

MSC – Mesenchymal stem cells

mTOR – Mammalian target of rapamycin

ND – Non-diabetic

Neu5Gc – N-Glycolylneuraminic acid

NEUROD1 – Neurogenic differentiation 1

NK – Natural killer cells

NKX6-1 – NKX homeobox 1

NOD – Non-obese diabetic

NODAT – New onset of diabetes after transplantation

NPI – Neonatal porcine islets

NSG – Non-obese diabetic severe combined immunodeficiency disease IL2rg^{null}

nT-Reg – Natural regulatory T-cells

OGTT – Oral glucose tolerance test

P4 – Progesterone

PBMC – peripheral blood mononuclear cells

PDE7B – Phosphodiesterase 7B

PDX1 – Pancreatic and duodenal homeobox 1

PERV – Porcine endogenous retrovirus

PLA-mPEG – Polylactic acid and methoxylated polyethylene glycol

p-MHC – Self-peptide major histocompatibility complex

PRL – Prolactin

PTDM – Post-transplant diabetes mellitus

PTLD – Post-transplant lymphoproliferative disease

PVPON – Poly(N-vinylpyrrolidone)

RAAS – Renin-angiotensin-aldosterone system

RAG – Recombination-activating genes

RI – Rat islet

ROS – Reactive oxygen species

SC-β – Stem cell-derived beta cell

SCID – Severed combined immunodeficiency disease

Sd(a) – Sid antigen

SLA – Swine leukocyte antigen

SNAP-25 – Synaptosomal Associated protein-25

SNP – Single nucleotide polymorphisms

SNT – Standard therapy/control group

SPK – Simultaneous pancreas-kidney transplant

T1D – Type 1 diabetes mellitus

T2D – Type 2 diabetes mellitus

TA – Tannic acid

TCF7L2 – Transcription factor 7-like 2

Th – T helper cells

THADA – Thyroid adenoma associated

TLR – Toll-like receptor

T-reg – Regulatory T-cells

UDCs – Universal donor cells

VAMP-2 – Vesicle associated membrane protein 2

ZnT8 – Zinc transporter 8

CHAPTER ONE

ISLET TRANSPLANTATION PROGRESS AND ALTERNATIVE

TRANSPLANTATION STRATEGIES

1-1 GENERAL INTRODUCTION:

Over the last forty years, the prevalence of diabetes has increased from 108 million people in 1980 to 422 million people in 2014 [75]. This number indicates that approximately 5.8% of the world's population was diabetic in 2014 [76]. The fact that all forms of diabetes contribute to more than 1.5 million deaths per year suggests the pressing nature of this disease [75]. In Canada, the prevalence of individuals diagnosed with all forms of diabetes in 2020 is 3.7 million people or approximately 10% of the Canadian population. Researchers expect that this number will reach 12% of the population by 2030. The evidence suggests that the global prevalence of diabetes will continue to rise, which consequently implies that the associated micro and macrovascular complications, such as strokes, heart attacks, kidney failure and non-traumatic leg and foot amputations, will increase as a result. Further, the financial burden of diabetes is a salient feature of the disease, as it has been shown that multiple daily insulin injections, insulin pump therapy and oral medications pay between $1,100 - $2,600 a year for out-of-pocket medical expenses. The cost implications for the Canadian Health Care system are even more alarming, where 3.8 billion dollars in direct costs pertaining to Type 1 Diabetes (T1D) alone have been incurred [77]. The rising rates of Canadian and global diabetes reveals that there is a need for a comprehensive and novel approach in confronting this critical issue. There is a vital and pressing need for a therapeutic approach and further research into the etiology of diabetes, but there is also a demand for treatment strategies that will be more cost-friendly for both the patient and the Canadian Health Care system. A well-rounded approach to diabetes should address the etiological, economic, and temporal implications of the disease.

The discovery of insulin by Banting, Best, Macleod, and Collip in 1921 has revolutionized our approach to the treatment of diabetes in general and T1D in particular.

However, since its discovery, insulin has only served as a treatment option and not a cure for diabetes [2]. The substantial rise in diabetes in recent decades may have been a response to the treatment options available to the public. There is now a vital need to develop and refine alternative strategies to treat T1D to maintain long-term insulin independence rates of patients and establish glycemic control, which cannot be established from exogenous insulin administration. The first clinical islet transplantation in 1977 was performed by Najarian et al. [104]. Unfortunately, none of the patients achieved sustained insulin independence beyond one year [103, 104]. In the subsequent years, advancements in surgical technique, islet purification strategies, and use of more potent immunosuppressive medications made islet transplantation a desired treatment option for T1D patients. However, the persistence of low insulin independence rates at one year made this treatment option unsuitable for the majority of T1D patients as the long-term benefits were not yet achieved [103]. Twenty-three years later in the year 2000, a group of researchers successfully treated T1D in seven consecutive patients undergoing islet transplantation. These patients achieved sustained insulin independence rates of 100% after one year and were treated with a glucocorticoid-free immunosuppressive regimen [82]. However, these patients were specifically selected as they have previously exhausted all previous treatment options, presented severe glycaemic lability and hypoglycemic unawareness. This illustrates that islet transplantation, as a treatment option, is limited to a small portion of the T1D patient population. Nonetheless, this protocol, later termed the Edmonton Protocol, was a landmark case in advancing our knowledge of finding a feasible treatment for T1D [82]. Islet transplant, as compared to pancreatic transplantation, is likely to carry a less substantial risk of mortality and is proven to be beneficial by maintaining metabolic control, normalizing glycosylated hemoglobin values and decreasing the need for exogenous insulin [82]. Although islet transplant now serves

as a possible treatment for T1D, there remain drawbacks to its approach and effectiveness. For islet transplant to successfully translate into clinical practice for a larger population of T1D patients with a higher success rate and to achieve prolonged periods of insulin independence, researchers must find solutions for the need of multiple islet donors, controlling islet graft rejection, and the need to reduce the usage of systemic immunosuppressive medications upon transplant.

Researchers are now expanding their efforts to use alternative islet sources, including xenogeneic and stem cell-derived islet sources, for widespread clinical practice. Porcine islets have been at the forefront of xenogeneic supplies, due to the fact that they are genetically malleable, and the structure of their secreted insulin closely resembles that of human insulin [57, 62]. In recent years, stem cells have gained significant attention for their ability to meet the need for multiple islet donors. Investigators are now looking at the efficacy of using allogeneic and autologous stem cell-derived islets, along with the pros and cons of each. Theoretically, the use of certain stem cells could pose as a method to decrease immunomodulation and reduce the need for systemic immunosuppression, thereby reducing the associated negative side effects [22, 13]. By using cellular genetic engineering methods such as CRISPR/Cas9 technology, stem cells and porcine islets could be produced with a lower immunogenicity and possibly enhanced insulin secretory capabilities [85, 188, 189].

Animal models have played significant contributions in allowing researchers to understand the pathophysiology of T1D in the past. The usage of humanized mouse models holds unlimited potential in allowing researchers to better understand the process of islet graft rejection using a wide array of β-cell sources and to develop and test novel strategies to delay graft rejection, in the context of a human immune system. However, there is a pressing need to

further refine this avenue of research to test novel therapeutic strategies on an established mouse model with a human immune system. In discussing islet transplantation and β-cell replacement therapy, it is important to first discuss the topic of diabetes, especially diabetes mellitus.

1-2 DIABETES MELLITUS:

Diabetes mellitus (DM) is a heterogeneous group of metabolic diseases (syndrome) characterized by an escalation in serum glucose levels due to its inability to enter the insulin-sensitive tissues, including hepatocytes, adipocytes, and myocytes [14]. The incidence of diabetes is rapidly growing worldwide, and it is estimated that over 400 million individuals are somehow affected by the complications of diabetes [13, 40]. It is expected that, in the next few years, the number of patients diagnosed with diabetes will dramatically increase [13]. In the United States alone, the projection of diabetes is expected to affect one third of the population [40].

The complexities of DM are the result of the disruption in maintaining normal glucose homeostasis, which in turn affects fat and protein metabolism. Pre and postprandial rise in the glucose levels, along with abnormality in protein and lipid metabolism is associated with a series of complex metabolic problems, the hallmark being diabetic ketoacidosis. Ketoacidosis is one of the earliest signs of undiagnosed diabetes and is evidence of the body's inability to regulate and balance bodily levels of ketones and insulin [31]. This has resulted in a vast number of micro and macrovascular complications such as retinopathy, neuropathy, nephropathy, stroke, coronary artery disease and peripheral artery disease [41]. These complications may stay chronically with patients during their lives and will progressively deteriorate later in their life translating into diminished life expectancy [6]. Though the overall average life expectancy of patients with

diabetes has improved from the 1960's, in 2002 it was predicted that American children who were diagnosed with diabetes at 10 years of age lived an average of 19 years less [130, 131].

Controlling the progression of the disease to decrease diabetic complications and thereby increasing the life span of patients are the first and foremost goals in treating diabetes [67]. Despite the vast knowledge about the pathogenesis of the disease, treatment options are limited. Most of the efforts in these regards are directed toward controlling blood sugar levels, which mostly minimize the micro and macrovascular complications of diabetes [15]. In this respect the most important factor is the in-time diagnosis and differentiation of disease. Other than clinical symptoms that are gold standard for diagnosis, measurement of blood sugar level in random and fasted states, oral glucose tolerance test (OGTT), quantification of hemoglobin A1C (HbA1c) and C-peptide for differentiation of T1D and Type 2 Diabetes (T2D) provide robust tools for the diagnosis of diabetes [145, 146].

Throughout the years, DM has been categorized into two main categories, Type 2 (Insulin-independent) and Type 1 (insulin-dependent), along with other less frequent categories, including Maturity onset of the young (MODY),) drug-induced diabetes, and gestational diabetes (GDM). The categorization of DM with differentiated pathogenesis and prognosis has dictated the treatment options.

1-2.1 Type 2 Diabetes:

Type 2 diabetes is more prevalent than T1D, and more than 90% of the diabetic cases are related to T2D [32]. T2D is mainly influenced by genetic factors, obesity, aging, and peripheral tissue resistance to insulin [40]. Based on the Genome Wide Association (GWAS) analysis, 139

common and 4 rare variants are associated with T2D, 42 (39 common and 3 rare variants) of which are independent of the known variants [86].

The etiology of T2D is related to either partial dysfunction of β-cells in the pancreas or lack of sensitivity to insulin in targeted tissues such as the liver, muscle or adipose tissue. In most cases, a combination of both factors may play a role in the disease [40]. Although the complications of both types of diabetes are similar in the long term, there are no or rare cases of ketoacidosis in T2D, unlike in T1D. Also, metabolic disorders in T2D can be less severe [32].

In recent years, epigenetic factors have been found to be involved in the progression of T2D. Many unidentified genes control the secretion, receptor production and action of insulin both on cellular levels and inside the cells. It is widely known that the methylation of DNA or modification of a gene by histones can alter the function of the gene by turning it on or off. This mechanism is involved in altering the function of many genes that are involved in the progression of T2D. These factors can describe the impaired secretion of insulin from β-cells. For instance, an increase in methylation of TCF7L2, THADA, KCNQ1, FTO, and IRS-1 genes and a decrease in methylation of CDKN1A and PDE7B genes are responsible for impaired insulin release in T2D [39]. Generally, T2D patients are not prospects for islet transplantation, since the large islet mass needed to overcome insulin resistance is not likely to be obtained from isolated islets [190]. Many factors contribute to the etiology of T2D, and the associated treatment options are broad. The general treatment for patients with T2D is a change in lifestyle factors, including a change in physical activity and diet [191]. Patients may also be prescribed Metformin to improve glycemic control through the enhancement of insulin sensitivity in the liver and muscle [192]. On the other hand, the treatment option for T1D remains limited to insulin therapy and transplantation [7].

1-2.2 Type 1 Diabetes:

T1D or Juvenile diabetes is mostly diagnosed in early adulthood or during puberty; however, some latent forms of T1D may occur later in life [30]. T1D is caused by the autoimmune destruction of pancreatic β-cells, which in turn affects the production of insulin and causes abnormality in glucose homeostasis. Extensive investigations have provided enough scientific proof for the involvement of immunological processes in the pathogenesis of T1D [27]. Evidence comes from the presence of white blood cell infiltration in the islets of Langerhans [195], the production of islet specific autoantibodies (IAA) in many T1D patients [196], and identical twin studies where the transplanted twin with T1D rejects the islet graft obtained from the other non-diabetic twin [197, 87]. Genetic predisposition, particularly polymorphism in class II Human Leukocyte Antigen (HLA) genes are believed to be involved in an autoimmune-mediated process that is associated with the destruction of β-cells [27, 132]. Specifically, individuals with HLA class II alleles for DR4, DQ8, and DQ2 have the highest risk in terms for developing T1D [87].

Current evidence suggests that T1D is a T-cell mediated autoimmune disease. Studies utilizing NOD mice suggest that CD4 and CD8 T-cells are implicated in the pathogenesis of T1D. Genetic defects in central tolerance permits islet autoreactive CD4 and CD8 T-cells to escape the thymus and reach the pancreatic lymph nodes. In this context, autoreactive CD4^{+} T-cells and IAAs interact with dendritic cells (DCs) presenting islet antigens. At this stage, CD4^{+} T-cells differentiate into Th1, Th2, Th17 (T-helper) and T-reg (regulatory T-cells). Th1 cells can further activate DCs and enhance antigen presentation to islet specific CD8^{+} T-cells. Transport of Th1 cells to the pancreas induce the secretion of pro-inflammatory cytokines such as IFN-α and IFN-γ, which are toxic to β-cells. The two latter cytokines can stimulate an orchestrated phenomenon in which macrophages in pancreatic islets produce reactive oxygen species (ROS)

as well as IL-1β, which contributes to the death of β-cells [87]. The inflammation in β-cells results in infiltration of CD8 T-cells and further destruction of β-cells [27, 87]. Autoreactive CD8 T-cells are activated through antigen presentation on major histocompatibility complex (MHC) class I and can moderate β-cell death directly through the release of perforin and granzyme B [87]. It has been suggested that the inflammation in β-cells can be dampened through the activity of natural regulatory T-cells (nT-reg). For this reason, T-reg cells are considered a therapeutic target in the treatment of T1D with considering strategies to increase T-reg cells [87, 88]. Human cord blood stem cells that are rich in T-reg cells are considered to increase the differentiation of naïve T-cells to T-reg cells [88].

It is well documented that insulin, glutamic acid decarboxylase-65 (GAD-65), islet antigen-2 and Zinc transporter-8 (ZnT8) work as autoantigens and promote the preclinical onset of T1D. Autoantigens are proteins or RNA complexes that may have improperly formed and are recognized by the immune system of autoimmune patients and targeted by autoantibodies. It is suggested that the production of autoantibodies may occur through the recognition of foreign antigens which may cross-react with self-antigens [193]. Autoantigens, on the other hand, are suggested to be the outcome of genetic mutations or neoantigen formation [194]. Insulin peptide A and B in humans is postulated to be essential targets for the immune system, resulting in the destruction of pancreatic β-cells. In many countries, IAA is used as the gold standard for predicting T1D. It has been observed that the presence of this antibody in early childhood strongly correlates with the progression of T1D. On the other hand, GAD, an enzyme that is involved in the process of neurotransmission and analgesia in the central nervous system (CNS), is released in the pancreatic β-cells. Between the two isoforms of GAD-65 and GAD-67, GAD-65 is more prevalently expressed in human islet β-cells. The presence of the autoantibody against GAD-65 in months to

years before the clinical onset of T1D in 70-80% of the patients could be used as a good prediction factor in the diagnosis of T1D. IA-2 (islet antigen-2) and its paralog IA-2β could also be used as another predictor of T1D. It is estimated that about 65% of the newly diagnosed patients with T1D have a high level of IA-2, and between 35-50% of these patients have a high level of IA-2β in their bodies [27]. ZnT8 is a member of SLC30A family of zinc transporters that are expressed on the surface of insulin secretory granules of pancreatic β-cells [89]. In comparison to the other autoantigens, ZnT8 is the most specific for β-cells. It has been demonstrated that expression of this protein is downregulated in the pancreatic β-cells of diabetic mice [90]. Studies in humans suggest that targeting ZnT8 specific T-cells can be a possible treatment option for the progression of T1D [27].

Although the role of immunological pathways in the pathogenesis of T1D is well characterized, there are some robust findings about the role of some environmental factors such as Coxsackievirus B (CVB) in the pathogenesis of the disease. Epidemiological findings demonstrate a strong correlation between CVB and T1D. CVB is shown to have tropism for pancreatic tissue leading to infection and destruction of β-cell and promotion of diabetes [28]. This tropism is related to the expression of Coxsackievirus Receptors in β-cells [29].

Viral infection from CVB promotes the recruitment of T-cells to the islets and local production of inflammatory cytokines and the interferon response [28, 252]. Animal studies have demonstrated that CVB is directly able to produce diabetes through infection of the pancreas. This may be due to a combination of genetic susceptibility, immune system response including inflammation and interferon response, as well as viral mutations that may make some viruses more prone to diabetic induction [28].

There is also evidence that indicates the role of Bcl-2 protein in controlling the intrinsic mitochondrial pathway of β-cell apoptosis induced by proinflammatory cytokines generated by CVB [91]. Other research indicates that molecular mimicry may also play a role in T1D induction, as the mechanism may confer cross-reactivity between viral and β-cell epitopes [253]. In one study, it was found that enterovirus infection can stimulate a cross-reactive immune system against IA-2 [253, 254].

Other than viral infections, some other environmental factors are assumed to participate in the development of T1D. For instance, there is a direct correlation between the weight of the mother at the time of pregnancy (BMI >30 and weight more than 200 lbs) and the development of T1D in offspring. Also, there is a direct correlation between overfeeding of children in their infancy stage or overnutrition of mothers prenatally and T1D [93].

One important complication with T1D is ketoacidosis, which is a severe metabolic disorder associated with intensive gluconeogenesis, proteolysis and lipolysis. Severe lipolysis increases the concentration of free fatty acids (FFAs), which in turn increases the level of ketogenesis and results in acidosis and electrolyte imbalance. Electrolyte disorder could also be aggravated due to the osmosis diuresis caused by an increased level of glucose in the blood. On the other hand, osmosis diuresis causes volume depletion, which activates catecholamine and cortisol release in the blood, which in turn increases the level of proteolysis and lipolysis in a vicious cycle and aggravates the ketoacidosis. Ketoacidosis is considered an emergency case in T1D and should be taken care of immediately [31].

Although patients with T1D are the current population who may be eligible for cell replacement therapies, there are other, less common forms of diabetes that will be also examined

in this section. These include maturity onset of the young (MODY), gestational diabetes, and drug-induced diabetes.

1-2.3 Other Types of Diabetes:

Maturity Onset of Diabetes of the Youngs (MODY):

MODY is a dominant, autosomal inherited non-insulin-dependent form of diabetes that was first diagnosed by American Physician Tattershall in 1974. It is estimated that between 1-5% of all DM cases are subtypes of MODY. From a clinical point of view, MODY is developed in adolescents under the age of 25 and is characterized by mild symptoms, which could be controlled by the administration of a low dose of sulfonylurea, or rarely insulin, in severe cases. Mutations in a number of genes are responsible for the different phenotypes of MODY. Patients with a mutation at HNF1A (Hepatocyte Nuclear Factor 1A) gene or subtype 3 MODY may be at risk of developing diabetes complications and may need to receive a low dose of sulfonylurea or GLP-1 (Glucagon-Like Peptide-1) analogues. Sulfonylurea acts on the β-cell by closing the K^+ channels, independently of ATP, thereby allowing depolarization, calcium influx, and subsequent insulin secretion [259]. GLP-1 analogues work to stimulate insulin secretion by acting on the GLP-1R on β-cells and decrease glucagon through α-cell modulation [260]. Mutation in the HNF4A (Hepatocyte Nuclear Factor 4A) gene is responsible for subtype 1 MODY. Patients with subtype 1 MODY may show different clinical features of diabetes, which may be aggravated in old ages or pregnancy and may need to receive insulin therapy. Mutation in Glucokinase (GLK) results in subtype 2 MODY. Patients, in this case, may experience mild clinical conditions that do not need any treatment. Other subtypes of MODY such as subtypes 4, 5, 6, 7, 9 and 11 are less important and are the result of mutations in different genes. One single feature in all subtypes of MODY is a defect in insulin production from β-cells rather than insulin resistance [42]. In theory, some MODY

patients may be eligible for islet transplantation as this is a genetic defect. MODY is a genetic form of diabetes, however diabetes can also occur in women during periods of gestation.

Gestational Diabetes:

Gestational diabetes mellitus (GDM) is a condition in which pregnant women exhibit abnormal blood glucose levels [71]. GDM is diagnosed at the time of pregnancy and often terminates after birth [72]. In normal instances of pregnancy, the placenta secretes diabetogenic hormones, including growth hormone (GH), corticotropin releasing hormone (CRH), placental lactogen (hPL), and progesterone (P4), which leads to insulin resistance. At the same time, the release of hPL and prolactin (PRL) results in enlargement (hyperplasia) of β-cells in order to overcome the insulin resistance. GDM occurs when the body is not able to overcome the associated insulin resistance [71]. As a result, both the mother and fetus experience diabetic complications since glucose readily crosses the placental barrier [71, 72].

Despite the normal blood sugar level of the mother after delivery, GDM can play a significant role in the global epidemic of T2D by increasing the chance of T2D in both mothers and children. Findings of a retrospective cohort study between the years of 1971 till 2003, using survival analysis on 5470 GMD patients and 783 control subjects indicated that mothers with GDM have 9.6 times more chance to develop T2D compared to control women [117]. Studies also indicated that GDM increases the chance of obesity, metabolic syndrome, and T2D in offspring between 2-8-fold [94].

The recommended treatment option for women who experience GDM is diet management and physical exercise. However, if these modifications fail to normalize the blood glucose level, insulin treatment will be required. Usually, patients can be treated with a combination of short and

intermediate-acting insulin analogs [72]. Shortly after delivery, the blood glucose levels of most women normalize, and their diabetic complications depart. To further understand the subtypes of diabetes, it is important to recognize the impact of different pharmacological agents on glucose homeostasis and possible diabetic complications.

Diabetogenic Drugs:

A variety of pharmacological agents alter glucose homeostasis and change the blood glucose level, which could be associated with diabetes in the long term. Although short term hyperglycemia by itself might not be transient, administration of pharmacological agents in patients with T1D or T2D could result in diabetic ketoacidosis in T1D and hyperosmolar hyperglycemic state (HHS) in T2D; both cases are hyperglycemic emergencies that need urgent attention.

The name of drug categories with the risk of hyperglycemia and, in some cases, diabetes is listed below [48]:

1. Beta-Blockers: These drugs may be prescribed to individuals with ischemic heart disease and hypertension [198]. However, many β-blockers, including propranolol, atenolol and metoprolol, take part in increasing blood sugar levels and exacerbate existing diabetes by impairing the release of insulin from pancreatic β-cells. Some other β-blockers such as carvedilol and nebivolol lack such metabolic activity and are more appropriate for their use in patients with diabetes.

2. Thiazide diuretics: These drugs are used to treat hypertension and they also decrease the insulin secretion from pancreatic β-cells by downregulating PPAR-γ (peroxisome prolif-erator-activated receptor γ). Further, they may activate the Renin-Angiotensin-Aldosterone

system (RAAS), which increases the aldosterone level and results in hyperglycemia. Hypokalemia induced by thiazides may further contribute to the hyperglycemic effect of these drugs.

3. Protease inhibitors: Protease inhibitors are part of the regiment for HIV treatment that may increase the chance of diabetes between 3-17%. These drugs induce hyperglycemia by promoting insulin resistance. Specifically, Ritonavir inhibits GLUT4 and induces hyperglycemia [48, 49, 50].

4. Corticosteroids: Corticosteroids are anti-inflammatory and immunosuppressive agents that are prescribed for a wide range of disorders [199]. Corticosteroids promote an increase in postprandial blood glucose level with no effect on the pre-prandial state. Mechanistically, this group of drugs neutralize the effect of insulin by elevating hepatic gluconeogenesis and are stated to be the most common cause of drug-induced DM [48, 199].

5. Calcineurin Inhibitors (CNIs): CNIs are immunosuppressive medications prescribed for a wide range of autoimmune conditions [200]. Sustained usage of Cyclosporine (CsA) and Tacrolimus elevate blood sugar level and may result in Posttransplant Diabetes Mellitus (PTDM). PTDM effect of these drugs is impacted by age, ethnicity, and concomitant usage of corticosteroids. It is hypothesized that the inhibition of pancreatic β-cells in releasing insulin is the most important factor in promoting hyperglycemia and diabetes [48].

Although these drugs are diabetogenic, some of these drugs, such as CNIs and corticosteroids, are given to patients undergoing pancreatic and/or islet transplantation. Paradoxically, although these drugs do contribute to islet dysfunction, many of these drugs such

as sirolimus, cyclosporine, tacrolimus, and corticosteroids are given to transplant patients to suppress their immune system to prevent the chance of graft rejection.

To understand the treatment strategies for T1D, it is important to have knowledge about the pancreas as well as insulin and glucose homeostasis.

1-3 THE PANCREAS:

In 1889, Minkowski and von Mering uncovered the link between the pancreas and diabetes, noting dogs that underwent a pancreatectomy produced higher than average amounts of urine [33]. This observation, later termed glycosuria, was found to be a symptom of diabetes [133]. Since this study, much research has been done to uncover how diabetes manifests.

The human pancreas is a single retroperitoneal organ (peritoneal in mice) consisting of an endocrine and an exocrine component. The endocrine portion contains three major types of islet cells that secrete insulin, glucagon, and somatostatin, that work together to maintain glucose homeostasis [32]. Insulin is a peptide hormone that is produced and secreted from pancreatic β-cells in the islets of Langerhans and controls the entry of glucose into muscle and adipose cells, as well as the storage of glucose in the liver in the form of glycogen. β-cells comprise ~55-75% of islet cells in the pancreas and insulin is the only hormone that lowers blood glucose level [95]. Glucagon is a 29 amino acid peptide hormone that is produced and secreted from pancreatic α-cells and regulates the hepatic release of glucose [96]. α-cells comprise ~20-40% of islet cells in the pancreas. It is well known that insulin and glucagon function antagonistically, and the varying ratios between insulin:glucagon determine the balance between anabolism (glucose and lipid storage) and catabolism (gluconeogenesis, glycogen, and lipid release) [95]. Somatostatin is produced and secreted in pancreatic δ-cells and comprises ~5% of islet cells in the pancreas.

Somatostatin is primarily involved in neuroendocrine function, but its release also inhibits the secretion of insulin and glucagon [95]. Another endocrine cell in the pancreas includes pancreatic polypeptide cells, which are secreted by F cells of the islets of Langerhans. Pancreatic polypeptide hormone is secreted postprandially to regulate digestion, delay gastric emptying, and restrict biliary flow [201-203]. There are other, less common endocrine cells in the islets, such as ε-cells, which produce ghrelin, the hormone responsible for inducing the stimulation of appetite [265]. On the other hand, the exocrine portion of the pancreas contains acinar cells and duct cells that secrete digestive enzymes and sodium bicarbonate into the duodenum, respectively. Acinar cells produce and secrete digestive enzymes, including proteases (trypsins that include: chymotrypsinogen A and B, proelastase, procarboxypeptidase), lipases (pancreatic triglyceride lipase), and amylase (pancreatic α-amylase) [134]. Bicarbonate, produced and secreted by epithelial cells in pancreatic ducts, neutralizes the acidic chyme entering into the duodenum [135]. The pancreas receives a majority of its blood supply from celiac arterial trunk and the superior mesenteric artery, and venous drainage occurs through the superior mesenteric vein and the splenic vein that join to become the hepatic portal vein [136]. The islet β-cells are arranged in pairs of 8-10 around a central capillary, with the granule containing poles facing towards the artery [137]. The exocrine gland accounts for around 85-95% of the cellular mass in the human pancreas [32]. It is estimated that there are approximately one million islets in the human pancreas, each with around 2000-3000 cells [273].

In a dysfunctional immune system, the ability of T and B lymphocytes to distinguish between the self and non-self is compromised. Thus, in T1D, the pancreatic islets are infiltrated with both lymphocytes from the adaptive and innate immune systems, contributing to what is known as insulitis. This process contributes to the progressive deterioration of β-cells, and

ultimately, the emergence of diabetic symptoms results once 70% of the islet cell mass has been eradicated [36]. The next section will examine insulin and glucose homeostasis to provide context for specific treatments of T1D.

1-4 INSULIN AND GLUCOSE HOMEOSTASIS:

In 1960, Anton Clemens successfully developed a tool that quantitatively measured blood glucose, Detrostix, which became available on the market in 1970 [138]. In addition to insulin therapy, reliable glucose monitoring along with exogenous insulin administrations became a widespread practice.

After the discovery of insulin by Banting, Macleod, Best, and Collip, many different types of insulin formulations have been developed for minimizing the hypoglycemic effects of DM. Examples are intermediate and long-acting insulins such as isophane insulin, insulin glargine, insulin detemir and insulin Degludec [2 - 4]. A change in the release kinetics of insulin from first degree to zero degrees by these formulations could control the hypoglycemic events; however, the practical experience in clinics does not show a hundred percent success [15, 16].

Insulin, in its active form, is a 51 amino acid peptide drug made of two chains (A and B) linked together by two intersubunits of sulfide bridges. Chain A consists of 21 amino acids and contains an intrasubunit disulphide bond between A6 and A11, whereas chain B consists of 30 amino acids [8, 147]. Primarily, insulin is made in the β-cells of islets of Langerhans in the pancreas as preproinsulin and then proinsulin. The latter undergoes proteolytic cleavage to form C-peptide and insulin in equal molar ratio [8]. Insulin is released in a pulsatile manner in response to an increase in postprandial blood glucose levels. However, in rare cases, a β-cell tumor may fire up the insulin secretion without postprandial glucose stimulation [16]. In certain cases, some amino

acids, free fatty acids, and hormones as well as immune mediators may intensify insulin secretion [17]. The oscillatory mechanism of insulin release has a period of 5-10 minutes in humans and is orchestrated by a pulsatory mechanism that results from an increased intracellular Ca^{2+} concentration [9]. Although the secretion of insulin is pulsatile, postprandial escalation in the blood glucose level intensifies the amplitude of oscillation. In other words, both the volume and rate of insulin secretion increase after a meal [10]. Mechanistically, glucose entrance to β-cells through Glucose Transporter 1 (GLUT1) in humans (GLUT2 in mice) and phosphorylation of glucose by a kinase enzyme called Glucokinase inside the cell initiate a process in which phosphorylated glucose changes to pyruvate and enters the mitochondria. Production of ATP in the mitochondria and its back entrance to the cytoplasm is stimulation for blocking the ATP-sensitive potassium channels, which inhibits the efflux of K^+ out of the cells. Depolarization of the membrane due to the overload of K_+ facilitates the opening of voltage-dependent Calcium channels and results in the flux of Ca^{2+} inside the cell. The rush of Ca^{2+} into the cell is associated with a process in which tethering, docking, priming and infusion of insulin loaded vesicles to the cell membrane initiate a process called exocytosis [11]. Degranulation of the insulin reservoir granules involves a couple of proteins, including Synaptosomal Associated protein-25 (SNAP-25), Syntaxin 1 and 4, Vesicle Associated Protein 2 (VAMP-2), synaptobrevins 1& 2, Munc-18 and Synaptotagmin [12, 18, 97].

It is important to mention the fact that insulin and glucose homeostasis can greatly be affected by central and peripheral nervous system regulation. The hypothalamus has been demonstrated to play a critical role in sensing energy reserves and hormonal balance to regulate food intake and endogenous glucose production. The role of the central nervous system to sense peripheral signals and respond appropriately promotes glucose homeostasis [261]. In addition, the effect of insulin can be affected from peripheral hormones such as cortisol, epinephrine, and

norepinephrine. High cortisol levels have been demonstrated to contribute towards insulin resistance in peripheral tissues [262]. In humans, increased epinephrine levels have demonstrated to elevate blood glucose levels but also slow down glucose clearance [263]. Although this effect may be transient, chronic elevation of glucocorticoid levels have been connected to the development of insulin resistance, which can lead to the development of further complications [264].

With a critical understanding of insulin and glucose homeostasis, we turn our attention to the discussion of different forms of treatments for T1D.

1-5 TREATMENTS OF TYPE 1 DIABETES:

As treatment options for T1D had been limited to exogenous insulin administration, researchers have sought out different methods in treating the disease. Treatment options fall into three basic categories, namely insulin therapy, transplantation, and immunosuppressive therapy. Though each method has its unique encounters, further research and the possible combination of therapies will help alleviate the challenges.

1-5.1 Insulin as a treatment of Type 1 Diabetes:

For nearly a century, exogenous insulin administration has remained the primary treatment option for DM. Throughout the late 1970's, the invention of the insulin pump allowed for a more physiological means of insulin regulation in comparison to exogenous intravenous insulin injections. The device, called the "artificial" or "bionic pancreas" has seen significant improvements in the past 50 years with different variations. One variation allows for continuous glucose monitoring through a feedback loop system, which has been demonstrated to be superior

in improving glucose control of patients in comparison to sensor-augmented pump therapy. As of 2019, this device has been approved for use in patients as an alternative source of exogenous insulin injection. Other variations of the bionic pancreas use a dual hormone approach that either employ glucagon or amylin monitoring and stabilization in addition to insulin regulation. However, the variations of the dual hormone approach have not yet been approved for use in patients since there are many challenges that have yet to be overcome. These challenges include the construction of dual-chambered infusion pumps and finding an efficient means to solubilize and stabilize glucagon for delivery. However, this approach can possibly improve the glycemic control of patients once the dual-hormone system becomes feasible [98].

The most important pitfall in insulin therapy, despite its effectiveness, is poor glucose homeostasis. This is evident in a famous study called the Diabetes Control and Complications Trial (DCCT). In this study, 278 volunteers were randomly assigned to either an intensive therapy group (INT) or standard therapy/control group (SNT) and their glucose homeostasis, hypoglycemia, and diabetic complications were compared. The INT group received a minimum of three daily insulin injections, whereas the SNT group received one or two daily insulin injections [69]. Results indicated that the INT group was more effective in reducing the micro and macrovascular complications of diabetes, although the SNT group receivers experienced more hypoglycemic events [67, 68].

It is well understood that metabolic complications in poor glucose homeostasis leads to micro and macrovascular problems. Microvascular complications include retinopathy, nephropathy, neuropathy, and end-stage renal disease, whereas macrovascular complications include stroke and myocardial infarction [41, 68]. It has been well established that the costs associated with increased frequency of hypoglycemic episodes is of less importance with the

decrease in homeostatic complications of glucose. Therefore, the conventional method currently in use is a mirror of the intensive insulin treatment [68].

Despite the effectiveness of insulin therapy, the prevalence of hypoglycemia, unlike hyperglycemia, is an emergency and demands prompt action. In one study, 7.1 % of the patients receiving insulin for the treatment of T1D and 7.3 % of the patients receiving insulin for the treatment of T2D experienced some degree of hypoglycemia [1]. Exogenous insulin administration does not mimic the oscillatory pulsatile mechanism, which is observed in endogenous insulin secretion [10]. This could be one factor that induces hypoglycemia during exogenous insulin administration.

Though insulin therapy remains the gold standard for the treatment of a majority of patients with diabetes, certain patients demonstrate a much more severe range of diabetic symptoms along with a decreased quality of life. As a result, patients who experience severe hypoglycemic unawareness often sought transplantation as alternative means of treatment [5]. The available options are pancreatic and islet transplantation, both of which will be examined in detail in the upcoming sections.

1-5.2 Pancreatic Transplant as a treatment of Type 1 Diabetes:

In 1966, Kelly and Lillehei were the first to demonstrate a successful pancreatic transplant on a human patient at the University of Minnesota hospital [34, 60]. As of 2016, more than 40,000 pancreatic transplants have been performed worldwide [35]. Despite the vast advancements in the area of organ transplantation, the use of potent immunosuppression is not ideal as it decreases the quality of life of patients and leads to further complications.

Although pancreatic transplant is a well-established protocol, the treatment option is limited to a select few patients who are at high risk of developing secondary diabetic complications and present severe and often fatal hypoglycemic unawareness [37]. Patients who are considered for a pancreatic transplant have experimented with all previous methods of diabetes treatment (i.e., numerous insulin injections per day, insulin pumps) with no indication of improvement [139]. Pancreatic transplantations are often performed in conjunction with kidney transplants. Usually, patients undergoing pancreatic transplant for T1D treatment are also presented with end-stage renal failure [33]. One study analyzed over 25,000 pancreatic transplants in the United States and discovered that the survival rates were 95% and 83% at one and five years, respectively [61]. In comparison to islet transplantation, pancreatic transplantation is a more invasive surgical procedure. Pancreatic transplantation meets the definition of an invasive procedure since it involves a method of access to the internal organs, requires instrumentation, and involves operator skill [99]. When combined with a simultaneous kidney transplant, pancreatic transplantation is correlated with significant mortality rates in the first 3 months [37, 139, 266]. Since islet transplantation is a safer procedure, patients are more often considered for islet over pancreatic transplantation when they have proper kidney function, given that they also have severe hypoglycemic unawareness [139]. During transplantation, patients must be provided with immunosuppressive drugs, often for the remainder of their lives, to prevent the chance of organ rejection [37]. In recent years however, advances in surgical technique, immunomodulation and donor selection have decreased the complications and contributed to a higher transplantation success rate and reduced the morbidity rates [100, 101].

A major drawback of chronic immunosuppressive therapies in pancreatic transplant patients is that it increases the chance of post-transplant malignancy [38] along with inducing

insulin resistance and destruction of pancreatic β-cells [44]. Gutierrez-Dalmau and Campistol [38] suggest that there is a higher incidence of malignant tumors in patients undergoing whole organ transplants compared to the wider population. One of the most common forms of post-transplant malignancies and a substantial cause of morbidity in pancreatic transplant patients is Post-Transplant Lymphoproliferative Disease (PTLD) [38]. On the other hand, the combination of immunosuppressive medications tacrolimus and mycophenolate mofetil has significantly increased the survival rate of patients undergoing pancreatic transplant as a means of treating T1D [60].

Pancreatic transplantation is a complex surgical procedure that has been refined over time. Kelly and Lillehei first introduced the technique of enteric drainage, which was at first correlated with a high level of surgical complications and organ rejections [34, 70]. Due to these issues, researchers sought out the new and improved bladder drainage technique, which allowed early detection of organ rejection and was associated with lower levels of surgical complications. However, this technique was related to complications in metabolic and urologic processes. The surgical technique that is most commonly used today is systemic venous and enteric drainage. With time, refinements in surgical techniques and the use of potent immunosuppressive medications have attenuated the organ rejection and mortality rate [70].

Although pancreatic transplant may be viewed as an effective means in treating T1D, patients who are eligible to receive this treatment are limited. With whole organ pancreatic transplantation, the 5-year graft survival rates in simultaneous pancreas-kidney (SPK) transplant patients are 70% [102]. Along with SPK, other forms of pancreatic transplantation include segmental pancreatic transplantation, and enteric drainage pancreatic transplantation [140] which is the current preferred method. Since pancreatic transplantation is limited in the patient selection

process, islet transplant may serve as a more suitable treatment for a larger proportion of diabetic patients.

1-5.3 Islet transplantation as a treatment of Type 1 Diabetes:

The first attempted clinical islet transplantation dates back to 1893 when Watson-Williams and Harsant attempted to transplant a piece of the sheep pancreas subcutaneously into a young boy with diabetes. The patient experienced temporary improvements in glycemia but unfortunately passed 3 days after [108, 103]. Almost 75 years later in 1967, a pioneer in islet transplantation, Dr. Paul E, Lacy, attempted the first islet isolation procedure in a rat pancreas using a collagenase-based isolation protocol [23]. This method was then successfully used in Rhesus monkeys a few years later [24]. In 1977, Najarian et al. [104] reported the first clinical islet allograft transplants in seven diabetic patients receiving azathioprine and corticosteroids. Unfortunately, these first clinical islet transplantation attempts were not successful since none of the patients achieved insulin independence [103, 104]. Between the years 1974 to 2000, there have been 450 attempts to treat T1D using islet allografts, and the majority of the immunosuppressive regimens consisted mainly of glucocorticoids, cyclosporine, and azathioprine with anti-lymphocyte serum induction [103]. Despite these efforts, less than 10% of patients remained insulin independent for longer than a year [103].

In the year 2000, a group of Edmonton researchers successfully achieved insulin independence in seven consecutive patients who were transplanted with allogeneic islets [82]. The Edmonton protocol differed from former islet transplantation procedures since they used a large islet mass (average of 11,547 +/- 1604 islet equivalents per kg of body weight), a high degree of islet potency, and a combination of three antirejection drugs consisting of sirolimus, tacrolimus

and daclizumab [19, 82, 84, 103]. All seven patients were able to discontinue exogenous insulin administration for a year [82]. However, the one- and five-year insulin independence rates in these patients were 100% and 10% respectively, indicating the need for an approach that would allow for prolonged insulin independence [103, 105]. This finding indicates that the clinical applications of islet transplantation have not been as successful in achieving the insulin independence rates of pancreatic transplantation, which is around 70% at 5-years post-transplant [102, 143]. As of 2016, the five-year insulin independence rates of islet transplantation patients have drastically improved with 50-70% of patients achieving sustained insulin independence, comparable to insulin independence rates of pancreatic transplantation [141]. Mechanistically, the drug combination used in the Edmonton Protocol works through different mechanisms and therefore, rejection of the allograft tissue is being controlled. From this combination, sirolimus inhibits the mTOR protein kinase involved in signal transduction and lymphocyte proliferation, tacrolimus inhibits the calcineurin and blocks the production of IL-2 and dacliximab is an anti-IL-2 receptor antibody which reduces the IL-2 driven T-cell proliferation during the acute rejection phase [21].

The conventional method used during islet transplant in clinical settings is as follows. The pancreas of cadaveric donors is procured, and the islets are isolated to obtain a clinical-grade level of purity using a combination of different steps. Once isolated and purified, the islets are transplanted into the liver via the hepatic portal vein, where they reside in the hepatic sinusoids. Once the islets have resided, they can secrete insulin and restore the normoglycemic state of patients. Although the hepatic portal vein is not the ideal location for transplantation, it is currently the gold standard in which the efficacy of other methods is evaluated on [65]. Other islet transplantation sites that have been experimentally evaluated on mouse models include the renal subcapsular space, spleen, striated skeletal muscle, omentum, testis, ocular chamber and the

pancreas [13]. Currently, research is being done to determine a more ideal location for islet transplantation.

Before examining alternative transplantation sites, it is important to consider the current challenges with islet transplantation.

1-6 TRANSPLANTATION CHALLENGES:

There are many challenges associated with pancreatic and islet transplants. In both instances, the primary challenges are related to the use of chronic immunosuppressive agents that are necessary to prevent transplant rejection. Additionally, with islet transplant, the issues entail the limited donor supply and the problem of islet vascularization post-transplant. Often with any organ transplant that is combined with immunosuppression comes the risk of developing DM and diabetic complications. This is due to the administration of diabetogenic drugs (ie. sirolimus, tacrolimus) that are necessary to prevent graft rejection. However, this exacerbates islet death upon transplant, contributing to lower prolonged insulin independence rates. Therefore, patients who receive pancreatic and/or islet transplantation may end up with diabetes again in the long haul. The fact that patients must undergo intensive and lifelong immunosuppressive therapy stresses the need for a more suitable immunosuppressive regimen. More specifically, researchers are moving from systemic immunosuppression and transitioning towards more localized immunosuppressive therapy post-transplant to lessen the negative consequences associated with immunosuppressive agents. Another method of reducing the reliance on chronic immunosuppression is through the induction of donor-specific tolerance (through eliminating donor-reactive T-cells and upregulating T-reg cells), allowing the patient to maintain proper immune system function [142]. This could possibly also be accomplished in the future with chimeric antigen receptor (CAR) T-cell therapy,

where the T-cells of patients can be modified to adjust the ratio of effector T-cells and T-reg cells, as well as introduce engineered TCRs to stimulate tolerance to the allo- and autoantigens [272].

Although islet transplantation is less invasive than pancreatic transplantation, it presents researchers with further challenges. On the one hand, there are a limited number of available donors, and as a result, an inadequate source of islet supplies. Therefore, the feasibility of this method is becoming more inaccessible. Currently, the ratio of required donors per recipient in islet transplantation is 2-4:1. In contrast to pancreatic transplant patients who receive a single organ from one donor, the necessity of obtaining multiple human islet donors is becoming more challenging. The procedures involved in obtaining islets from multiple donors have proven to be another issue that increases the complexity of donation. At the moment, a large number of cells (>900,000 islet equivalents) are required for clinical islet transplantation, which cannot be acquired from one donor. This is mostly due to the low level of cell recovery from the isolated tissues by current methods. The human pancreas contains $0.3 - 1.5 \times 10^6$ islet cells per pancreas, and only 30-50% of them are retrievable. Furthermore, only 65% of human islets are viable [21]. It is estimated that 50-70% of the transplanted cells undergo apoptosis during the process of isolation, culture, and the peri-transplant period [21, 148 - 150]. The physical processes involved in isolating the islets, which include separation from the native microenvironment and the subsequent devascularisation, denervation, and hypoxia, contribute to the loss of cells upon transplantation. Therefore, nourishing these isolated cells in culture to allow for recovery, along with using an established revascularization procedure may partly diminish the chance of allorejection [21].

To solve the devascularization problem, a proper implantation site should be selected. This problem is still one of the main concerns in islet transplantation therapy. The optimal transplantation site must have a high level of blood flow and be immunologically privileged to

minimize transplantation rejection [13]. Currently, the intraportal infusion site remains the benchmark by which all future transplantation locations are compared [13, 65]. The main challenge in choosing this site is the need for surgery, which increases the chance of bleeding in the intraperitoneal cavity from the liver surface following the withdrawal of the portal catheter. These technical issues could potentially be resolved by using some precautions such as occlusion and obliteration of the catheter track using soluble homeostatic paste agents and delivering heparin in appropriate amounts to the portal vein. Other than the use of proper surgical techniques, embolization and trapping of the transplanted cells within the portal sinusoidal capillaries has remained the main concern [13]. This issue calls for more research concerning finding an extrahepatic tissue suitable for islet transplantation. Unfortunately, extensive research to seek another proper tissue as the site of islet transplantation has resulted in unsatisfactory outcomes.

One other main issue in islet transplantation could be the occurrence of a reaction called instant blood mediated inflammatory reaction (IBMIR), which is the result of the exposure of tissue factor on the islet surface to blood cells. Such exposure attracts platelets to the islet surface and activates the cascade of a blood clot, which in turn recruits inflammatory cells, resulting in inflammation. During the first hour post-transplantation, >50% of the transplanted islet mass is destroyed by the innate immune system. The IBMIR contributes to significant β-cell destruction and amplifies cell-mediated rejection to transplanted islets [143, 148 - 150]. To protect against islet destruction, precoating the islets with protective macromolecules or administration of an anticoagulant such as heparin or dextran sulfate can improve islet survival by downregulating the IBMIR, at least in experimental settings [13].

Despite these drawbacks, cell replacement therapy is still the gold standard in terms of transplantation, which is a definite and unambiguous therapy for patients undergoing life-threatening hypoglycemic unawareness due to T1D [5].

Possibly the most important issue that arises during transplantation of either islets or the pancreas is the need for chronic and systemic immunosuppression. Since cadaveric islets are extracted from multiple donors, there is a need for chronic immunosuppressive therapy to decrease the chance of graft rejection. If immunosuppressive medication is not administered in transplant patients, there is the high risk of auto and allo-immune mediated graft rejection. Pioneering of the Edmonton Protocol was a major advancement in the field of islet transplantation since the researchers employed a ground-breaking glucocorticoid-free immunosuppressive therapy that allowed for sustained graft survival and functionality within 1 year [82]. In the case of pancreatic (whole-organ) transplant, although there is a less rigorous need to match the class I HLA subtypes, transplant patients still require prolonged immunosuppression [22]. There is an urgent need to develop a more suitable immunosuppressive program that eradicates many of the unwanted side effects.

The use of immunosuppressive medications is associated with critical side effects that negatively affect the patient's quality of life. For instance, administration of sirolimus and tacrolimus in combination increases the chance of nephrotoxicity and administration of sirolimus alone in female patients increases the occurrence of ovarian cysts [21, 82]. Although theoretical usage of targeted immunosuppressive drug delivery for sirolimus and cyclosporine seem to shed some light on this dark feature of immunosuppressive therapy, clinical usage of these new formulations is still far from reality [20, 25].

A critical issue that may arise from the use of immunosuppressive agents after transplant is Posttransplant Diabetes Mellitus (PTDM), previously known as New Onset of Diabetes after Transplantation (NODAT). The prevalence of PTDM is increasing over time and differs among the diverse age groups. For instance, pediatric patients receiving immunosuppression showed 20% of PTDM during the period of 1996-1999. This number indicates a ~10-fold increase when compared with the prevalence of disease between 1986-1990 (2.1%). Two large cohort studies identified a huge divergence between the pediatric and adults receiving immunosuppression after renal transplantation (2.6-7.1% in children versus 4.7-11.5% in adults) [43]. Close attention to these differences can reveal the pharmacokinetic differences between the pediatric and adult populations in which absorption, distribution, metabolism and excretion of drugs are different. A lower level of absorption can be observed in pediatric patients; however, clearance and drug metabolism could be slower in adults [43, 267].

PTDM most likely occurs in patients who have pre-existing T2D risk factors such as age, obesity, family history, race, and ethnicity. In terms of genes, Hispanic patients with single nucleotide polymorphisms (SNPs) for Insulin Receptor Substrate-1 (IRS-1) and Hepatocyte Nuclear Factor 4 (HNF4) are more prone to developing PTDM after kidney transplantation. The existence of some other SNPs that increase the risk of T2D in individuals receiving transplants may increase the chance of PTDM. These SNPs include TCF7L2, KCNJ11-Kir6.2, and some but not all variants of KCNQ1. Individuals who carry multiple SNPs may have a higher chance of developing PTDM. It is also known that carrying multiple IL genes, particularly IL-2, IL-7R (IL-7 receptor), IL-17R, IL-1β and IL-4, makes one more prone to developing PTDM [44].

Among different types of immunosuppressants, the usage of corticosteroids is associated with the highest level of PTDM. By increasing hepatic gluconeogenesis, stimulating appetite and

increasing weight gain as well as inducing or worsening pre-existing insulin resistance in peripheral tissues, corticosteroids increase the chance of PTDM in patients receiving them. Early withdrawal of corticosteroids or being on a low maintenance dose of these drugs rule out the development of PTDM after organ transplants [44]. *In vitro* incubation of murine β-cells or human cell lines with dexamethasone revealed an increase in the expression of α2-adrenergic receptors [55], decrease in the level of GLUT2 protein at β-cell plasma membranes [52], downregulation of glucokinase in mRNA level [53], and increase in β-cell apoptosis through corticosteroid receptors [54]. These factors all participate in PTDM following corticosteroid administration. It is well documented that administration of GLP-1 receptor analogues such as liraglutide and Exendin-4 (Exenatide) can inhibit the apoptosis of β-cells [51, 54] and probably reverse the diabetogenic effect of glucocorticoids. The clinical importance of such interactions is yet to be identified.

Strong evidence documents the participation of Calcineurin Inhibitors (Cyclosporine and Tacrolimus) and inhibitors of the mammalian target of rapamycin (mTOR-Inhibitors) (Rapamycin or Sirolimus) in PTDM. Larsen et al. [45] suggested a dose-dependent effect of both tacrolimus and sirolimus on glucose metabolism in Sprague Dawley rats. In comparison between tacrolimus and cyclosporine for their PTDM effect in a randomized clinical trial study, those who received tacrolimus were more prone to developing PTDM compared to those who received cyclosporine [46].

The mechanism involved in the development of PTDM by tacrolimus could be related to the increase in the resistance of cells to insulin along with no hyperinsulinemia suggesting loss of insulin secretion. Tacrolimus and cyclosporine were also found to reduce the activity of glucokinase, which in turn decrease the expression of the insulin gene. Further evidence suggests that tacrolimus can result in the induction of apoptosis in β-cells [47].

The most important factor in the diabetogenic effect of tacrolimus is found to be related to its dose. A decrease in the administered dose of tacrolimus is associated with a significant reduction in developing PTDM. The incidence of PTDM between 1999-2004 shows a 15% decrease, which is mostly related to using protocols that reduce the dose of tacrolimus from 9.5 to 6.4 ng/mL [56]. A comparison of all the immunosuppressive drugs for their diabetogenic effects has shown that mycophenolate mofetil and azathioprine have a limited effect on blood sugar levels, and their potency in inducing PTDM is less in comparison with other drugs [44].

The challenges for decreasing the diabetogenic effect of immunosuppressive drugs along with maximizing their anti-rejection effects have motivated scientists to develop new formulations with altered pharmacokinetics. One such application is the usage of micellular delivery of drugs to purposefully modify drug distribution towards target tissue(s). Despite vast research in this regard, the development of a desirable formulation is still in its infancy stage, and clinical usage of these new formulations is still far from reality. Theoretically, the use of a micellular drug delivery system can allow the controlled release of immunosuppressant medications, such as cyclosporine and sirolimus, to elicit localized immunomodulation. Such a procedure has advantageous effects over traditional immunosuppressive therapy that induces systemic immunosuppression and results in unwanted side effects. The ability to elicit localized immunosuppression in target tissues can possibly lessen the adverse effects of cyclosporine, including nephrotoxicity, hypertension, neurotoxicity, hyperglycemia, and gastrointestinal upset among other effects [73]. Further, diabetogenic drugs damage islets upon transplant, which creates a strain on the number of necessary cadaveric islet donors. The ability to control the kinetic release rate and delivery of drugs to target tissues has the potential to significantly decrease the adverse side effects associated with the use of these drugs, decreasing the need for multiple islet donors

and increasing patient quality of life. Although this procedure is far from clinical translation, it can potentially increase the span of time in which patients are in euglycemia.

In one study, cyclosporine-A was encapsulated in nanoparticles made of a mixture of Polylactic acid and methoxylated polyethylene glycol (PLA-mPEG) using a nanoprecipitation method. After determination of the physiochemical properties of nanoparticles (size, stability, and release of cyclosporine at different time points), the capability of prepared nanoparticles in suppressing T-cell proliferation as well as suppression in inflammatory cytokine was measured. In parallel, the internalization of cyclosporine nanoparticles by dendritic cells and their delivery to T-cells was measured in *in vitro* and *in vivo* scales (lymph nodes). This study demonstrated that cyclosporine nanoparticles not only inhibit alloreactive T-cells but also provide less organ toxicity [20]. This study can be a starting point to provide an efficient nano or microsystem for the delivery of immunosuppressant in both preclinical and clinical models.

In another study, local delivery of rapamycin microparticles within the anterior chamber of the eye (ACE) was demonstrated to be able to enhance the survival of grafts following an islet transplantation in the same location of the other eye in the same experimental animals. The investigators postulated that local delivery of immunosuppressants can protect the graft recipients from the harsh adverse effect of immunosuppressants [81].

Micellular delivery of antidiabetogenic drugs is not the only method in eliciting localized immunosuppression. Coating islet cells with a combination of poly(N-vinylpyrrolidone) (PVPON) and tannic acid (TA) prior to transplantation can reduce ROS and act as anti-inflammatory agents. Using this approach, scientists were able to elicit localized immunosuppression as indicated by a decrease in permeation of immune cells at the site of transplant [74].

There have been many challenges with pancreatic and islet transplantation. In the next section, the topic of immunity and graft rejection will be explored in further detail.

1-7 IMMUNITY AND GRAFT REJECTION:

The mechanisms of allo- and autoimmune mediate graft rejection are not completely understood in humans. During allogeneic islet transplant in autoimmune recipients, it is unclear whether the primary causes of graft rejection are due to the auto- or alloimmune response. Seetharam et al. [92] suggest that solid organ transplantation appears to involve the alloimmune response followed by the de-novo formation of the autoimmune response. Further research regarding immune mechanisms associated with cellular transplantation will provide further insight into the primary immune response involved.

Animal models of xeno- and allograft rejection have allowed scientists to study mechanisms of islet graft rejection and have broadened our knowledge of the immune system. To understand the basis of graft rejection, we must first examine mechanisms of auto- and alloimmunity, which will be discussed in order. Prior to understanding immune mechanisms associated with graft rejection, it is important to discuss antigen presentation.

Under normal instances, MHC class II molecules present antigens to CD4$^+$ T-cells, and the expression of these class II molecules are restricted to antigen presenting cells (APCs) such as dendritic cells, macrophages, and B-cells. In contrast, MHC class I molecules, which are expressed on all nucleated cells, present antigens to CD8$^+$T-cells [255]. Naïve CD8$^+$ T-cell survival involves identifying self-peptide MHC (p-MHC) and IL7, and naïve CD4$^+$ T-cell survival involves identifying IL-7 only [166].

Under autoimmune conditions, defective T-reg cells may have increased in number or increased in their immunosuppressive function. This is termed T-cell homeostatic proliferation and has been described as a mechanism that may induce autoimmunity. In lymphopenic states, remaining T-cells proliferate to establish internal equilibrium through the process of acute homeostatic proliferation. In this state, $CD4^+$ and $CD8^+$ T-cells proliferate through the recognition of self p-MHC and IL-7 [166]. This cytokine-dependent expansion process is largely controlled by cytokines of the common gamma chain receptor family [167]. During this process, some of the naïve T-cells that proliferate obtain indicators expressed by antigen-activated T-cells. Studies have shown that T-cells that have a higher affinity for the self are favoured [166]. Further, studies have shown that T-cells that have undergone homeostatic proliferation are similar in function to antigen-expanded memory cells [167]. Other studies have shown that lymphopenia may be associated with autoimmunity in animals and humans, contributing towards T1D. Evidently, mice which have undergone thymectomies shortly after birth developed T1D at an accelerated rate compared to mice undergone sham-thymectomies. This indicates that thymectomies performed in the early weaning period in mice had an extensive effect on immune system defects [168]. Further, Monti et al. [167] found that, compared to their pre-transplant status, patients who have undergone islet transplant had reduced circulating lymphocytes due to an increase in IL-7 and IL-15 immediately post-transplant. The researchers found that these conditions promoted homeostatic proliferation that led to the expansion of autoantigen-specific T-cells. This indicates that defective T-cell development and signalling contribute towards the development of autoimmunity [167].

During transplantation of allogeneic material, the alloimmune response predominates in response to transplantation of HLA-mismatched products. The alloimmune response is adaptive

and specific, mediated by T-cells that recognize alloantigens on the surface of transplanted cells and tissues. Alloantigens presented on MHC class I and class II molecules are implicated in producing the strongest T-cell mediated immune response during instances of histoincompatiblity [169]. Allorecognition can be subcategorized into three pathways: direct, indirect, and semidirect. In the direct pathway, alloantigens are directly presented by the donor's APCs to host T-cells [170]. Further, this pathway is associated with an acute rejection response, which is described as a slight deterioration of allograft function and, in most cases, an absence of histological examination [171]. In contrast, the indirect pathway of antigen recognition occurs when host T-cells recognize self APCs containing donor MHC antigens on host-HLA molecules. This pathway is associated with chronic graft rejection since donor APCs are replaced by that of the host over time [170]. In the semi-direct pathway, direct pathway T-cells can recognize allogeneic MHC molecules after being transferred from the surface of donor cells to the surface of recipient cells via exosomes [169 ,172]. However, the clinical significance of the semi-direct pathway is yet to be established in transplantation procedures [169]. T-cell mediated allorejection is facilitated by immunological memory and specificity. These traits can best be exemplified during an instance of second set rejection, where re-transplantation of the same allogeneic material in the recipient results in a more rapid rate of rejection. In contrast, first set rejection is slow and gradual in development and occurs when the recipient receives foreign cells or tissues during an initial encounter [169]. In summary, rejection of allogeneic human islets could occur through the direct, indirect, and semi-direct pathways and involves both CD4$^+$ and CD8$^+$ T-cells [169]. On the other hand, rejection of porcine islets predominantly involves the indirect pathway of antigen recognition and CD4$^+$ T-cells [174].

Although the mechanisms of porcine islet rejection are similar to that of human islets, differences exist in the mechanisms that predominate during graft rejection. In the xenotransplantation of porcine islets, it has been demonstrated that human CD4^{+} T-cells and the indirect pathway of antigen recognition predominates in the early stages of graft rejection [174 - 176]. In an early study performed by Friedman and colleagues [175], immunodeficient C57BL/6 mice transplanted with porcine FICCs and reconstituted with human peripheral blood mononuclear cells (PBMCs) displayed graft destruction predominantly through the actions of CD4^{+} T-cells. Friedman et al. [175] also showed that CD8^{+} T-cells were absent during the early stages of graft destruction but increased in number at later time points. Kobayashi et al. [174] demonstrated that *rag*-/- (C57BL/6) mice transplanted with microencapsulated NPIs displayed rejection when given CD4^{+} but not CD8^{+} T-cells from naïve B6 mice. In another study using C57BL/6 strain-derived MHC class II Aa gene-deficient mice transplanted with fetal pig pancreas (FPP) or rat islets (RI) displayed CD8^{+} T-cell dependent graft rejection in both groups. However, the kinetics of graft rejection of RI were faster than the rejection of FPPs, which may be due to the low expression of MHC class I molecules on FPPs. The authors conclude that the presence of CD8^{+} T-cell mediated graft destruction in these mice does not signify that this is the predominant mechanism of graft destruction. It is possible that CD8^{+} T-cells may have secreted cytokines and chemokines that recruited NK cells and macrophages, which have aided in the destruction of the grafts [177]. Together, these studies evidently indicate that CD4^{+} T-cells predominantly play a role in the initial stages of graft rejection, and that CD8^{+} T-cells also play a possible role in graft rejection at later time points [174 – 177]. Further research should focus on clarifying the role of CD8^{+} T-cells in graft rejection.

Graft rejection may cause long-term deterioration of islet graft function. The clinical manifestations of islet graft loss present in the form of loss of glycemic control. Histological analysis of islet graft loss has been a difficult feat as infused islets are distributed throughout the whole liver in transplant patients. Therefore, studies of islet graft rejection in human patient's post-mortem have been limited [173]. Researchers are currently investigating alternative islet transplantation sites to achieve more optimal results in patients.

1-8 ALTERNATIVE TRANSPLANTATION SITES:

Currently, the portal vein serves as the gold standard for clinical islet transplantation. However, the prevalence of the IBMIR, thrombosis, and hepatic ischemia results in the loss of up to 70% of transplanted islet mass within the first 48 hours [143, 148 - 150]. As a result, patients often require multiple islet infusions and lifelong chronic immunosuppression to prevent graft rejection. Although hepatic portal islet infusion allows for ease of access through cannulation and near physiological insulin secretion, the use of potent oral immunosuppressive agents in the long-term expose islets to potentially toxic levels in the portal circulation [151, 152]. In addition, it has been suggested that the function of α-cells are sub-optimal in the hepatic sinusoids compared to insulin producing cells [151]. This must be considered when transplanting stem cell-derived islets, as they secrete not only insulin but also glucagon [125]. For islet transplantation to become a more successful means of restoring glucose homeostasis in the long term, a transplantation site that is immunologically privileged, highly vascularized, neurologically innervated, and is clinically applicable must be discovered [150].

Previously, multiple transplantation sites have been explored as potential alternatives to the portal vein. These include the kidney capsule, spleen, pancreas, and intramuscular and subcutaneous spaces [150].

The kidney subcapsular space may pose an advantage in clinical islet transplantation when combined with renal transplantation using the same donor. In this case, islets could potentially be transplanted under the kidney capsule *ex vivo* and subsequently the kidney can be transplanted into the recipient. However, the kidney subcapsule is not considered an ideal site for islet transplantation due to several challenges. Islet transplantation under the kidney subcapsule would involve a laparotomy that is far more invasive than islet transfusion in the portal vein. In addition, the oxygen tension of islets under the kidney subcapsule is low and a higher number of islets may need to be transplanted to establish euglycemia in human patients. In a non-human primate model, it has been shown that the portal vein is a more ideal transplantation site compared to the renal subcapsule due to the ease of access and lower number of islets needed to establish euglycemia [150]. Although the kidney capsule is not the ideal location for clinical islet transplantation, this area is often widely used in rodents in experimental islet transplantation as it allows for histological analysis and explantation studies [153].

Islets transplanted into the spleen would be exposed to a good vascular network which would allow insulin delivery to the hepatic portal vein. Along with immune regulation, the spleen has been implicated to be involved in the regeneration of islets post-transplant [154]. In one study, the spleen of mice was found to contain islet stem cells that could potentially treat diabetes [155]. In another study, intrasplenic islets transplanted under the kidney subcapsule of mice displayed proliferation and expression of insulin. In this sense, the spleen could not only pose as a potential transplantation site, but also a possible islet source [156]. However, the risk of

bleeding remains a major barrier to utilizing the spleen as a transplantation site in clinical practice [150].

The pancreas is an attractive alternative to the portal vein, as this location is the native habitat of islets. Studies in animal models have shown that the pancreatic site provides high oxygen tension and minimal inflammation resulting in good graft survival. However, translation to clinical practice has not become feasible due to the surgical complications that may arise during this procedure [157]. Further, Cantarelli and Piemonti [157] suggest that there may be the possibility of accelerated T-cell mediated destruction of islets transplanted in the pancreas in patients with type 1 diabetes.

The intramuscular and subcutaneous spaces have also been investigated as potential transplant sites. Due to the ease of accessibility for implantation and biopsies, this site was posed as a promising alternative compared to hepatic islet transplantation [150]. In pilot studies of human clinical trials, islets transplanted into the intramuscular space provided better revascularization compared to portal vein infusion [157, 158]. Yet, fibrosis and necrosis prevented intramuscular-transplanted islets from functioning in the long term [157, 159]. In addition, hypoxia and lack of early neovascularization are other factors that contribute to decreased islet graft survival. In animal models, it has been demonstrated that angiogenesis in the intramuscular space prior to transplantation could contribute to improved and accelerated vascularization of islets post-transplant [150, 160]. Further, Pepper et al. [161] successfully demonstrated neovascularization and subsequent euglycemia in mice that were subcutaneously prevascularized prior to transplant with human islets or syngeneic mouse islets. Although the intramuscular and subcutaneous sites are attractive alternatives to portal vein infusion, much experimental research still needs to be done.

Recently, brown adipose tissue (BAT) has also gained significant attention due to its thermogenic property, highly vascular and innervated nature, and presence of anti-inflammatory phenotype. In a one recent study, Kepple and colleagues [162] conducted human islet transplants under the kidney capsule or BAT deposits of NOD mice. They found that islet transplanted into the BAT maintained euglycemia for a significantly longer amount of time and delayed autoimmune-mediated graft rejection compared to the islets transplanted under the kidney capsule. They also found CD31$^+$ vasculature situated next to transplanted islets in BAT. Further, islets transplanted in BAT displayed reductions in proinflammatory IFN-γ^+ CD4$^+$ T-cells and increases in anti-inflammatory CD4$^+$ T-reg cells [162]. However, the BAT as an alternative transplantation site in humans and larger animal models have yet to be investigated.

Along with the investigation of alternative transplant sites, researchers are also evaluating alternative β-cell sources to overcome the limited human donor pool.

1-9 ALTERNATIVE β-CELL SOURCES:

The limited supply of cadaveric islet donors along with the heterogeneity in islet isolation procedures creates many challenges with islet transplantation. Although the same clinical procedure and the same immunosuppressive drugs may have been used across clinics, the lack of adherence to a standardized islet isolation protocol contributes to the wide variability in outcomes and the reduced potential to compare results [106]. Due to the limited supply of cadaveric islet sources, researchers are turning to novel approaches as potential means in treating T1D. These sources include, but are not limited to, xenogeneic and stem cells sources. Though both methods have been translated to clinical settings, the procedures must be standardized to achieve optimal potential.

1-9.1 Xenogeneic Sources:

One of the main problems that researchers face with allogeneic (same species) islet transplantation is the limited supply of β-cells from cadaveric donors. To overcome this problem, one alternative is to propose the use of islets from a xenogeneic source. In 1893, Watson-Williams and Harsant first attempted to treat a young diabetic male by performing a subcutaneous implantation of a piece of the sheep pancreas. Although the patient experienced a short-term improvement in glycosuria, the patient did not survive for long [108]. Since this event, many other xenogeneic islet sources have been considered, including insulin producing cells from fish [110], rabbit [111], bovine [112], and porcine [109]. Out of all these sources, the best candidate to date has been porcine islets [107]. The reason that pig islets are selected as a candidate source is rationalized by the advantages that it offers in comparison to other animal tissues. Using a porcine supply of islets may allow for genetic modification and are more viable compared to other animal tissues [62]. Further, pigs are inexpensive, readily available, have rapid breeding potential, and produce numerous offspring of large size [107]. Also, insulin produced by porcine islets are similar to that of humans, with a difference in only a single amino acid [62]. Genetic modification of porcine islets may allow for enhanced engraftment and insulin secretion ability [57, 113]. The modification of porcine islets can reduce the potential risk of cross-species transmission of porcine endogenous retroviruses to humans (PERV) [113]. Furthermore, the ability to raise pigs in a controlled environment may reduce the possibility of transmitting this disease to humans. Porcine islets can be classified into 5 broad categories, namely fetal, perinatal, neonatal, juvenile, and adult sources; each presenting their own unique

advantages and disadvantages. It should be noted that three main groups of porcine islets, namely fetal, neonatal, and adult have been studied more extensively.

Islets extracted from the pancreas of fetal porcine contain immature β-cells that can be differentiated to produce fetal porcine islet-like cell clusters (FICCs) [107]. Fetal pancreatic β-cells on their own lack glucose-induced insulin secretion. However, increasing intracellular cAMP levels stimulates the release of insulin secretion from porcine fetal β-cells [107, 114]. Korsgren et al. [114] have developed a technique to produce FICCs that are able to secrete insulin. The primary advantage of using fetal porcine sources is that it is a cost-effective approach. Since raising pigs in a controlled environment is an expensive procedure, the reduced time frame of raising both fetal and even neonatal pigs make it a cost-effective approach compared to raising adult pigs [115]. The main disadvantage in the usage of FICCs is that they may take several months to reverse the hyperglycemic state *in vivo.* Further, a large number of donors would be necessary to treat a single human patient, making this an inefficient source [107]. It is estimated that islets extracted from around 100 fetal pigs are necessary to treat an average 70 kg patient [107, 114]. Thus, other porcine supplies have been considered for more effective treatment options.

Neonatal porcine islets (NPIs) have gained significant attention over the years as a plausible source of islets. NPIs can be compared with cadaveric human islets to demonstrate their potential in treating T1D. Human islets are particularly sensitive to hypoxic environments and inflammatory damage, reducing their ability to survive for prolonged periods post-transplant [78]. In fact, more than 60% of human islets are destroyed minutes to hours post-transplant [13]. Further, the procedures necessary to extract cadaveric islets activate metabolic pathways that destroy β-cells, further reducing their yield [63]. Therefore, many of these cells may not be as

viable during transplant. On the other hand, neonatal porcine islets (NPIs) have displayed the ability to recover from the detrimental effects of hypoxia. One study revealed that when NPIs were exposed to a hypoxic environment for 24 hours followed by a reoxygenation period of 24 hours, the initial decline in the insulin secretory capacity returned to baseline [78]. In 1996, Korbutt et al. [109] developed a technique for the isolation of NPIs, obtaining a 35% islet yield or approximately 50,000 islets. There are numerous advantages of using NPIs. Primarily, NPIs may translate into a higher functionality of islets due to their ability to overcome the lack of vascularization immediately post-transplant [59]. Secondly, similar to fetal porcine islets, raising NPIs in a controlled environment is cost effective compared to raising adult pigs [115]. Vanderschelden et al. [116] have studied the cost and scalability of neonatal and juvenile piglets in comparison to adult porcine sources. Specifically, raising of neonatal piglet accounts to $0.02/IEQ (islet equivalent), juvenile piglets accounts to $0.04/IEQ, whereas adult porcine islets accounts to $0.09/IEQ [116]. Further, reducing the time frame in which porcine islets are maintained and controlled reduces the probability of cross-species contamination of PERV [113]. In addition to its cost effectiveness, neonatal porcine islets are easily isolated, require little if any purification, are resistant to hypoxia, and demonstrate proliferative abilities *in vitro* and *in vivo* [115]. Although the usage of NPIs have certain advantages, they present major challenges that need to be overcome. The first disadvantage is that a large number of neonatal piglets are required per patient (around 70 neonatal porcine donors per 70 kg patient). Second, the expression of surface antigens on NPIs increases the possibility of tissue xenorejection [107]. Although the issue may be overcome by genetic engineering of porcine islets in the future, this is the main concern of using porcine islet transplant in humans [113]. More specifically, porcine islets contain antigens such as αGal, Neu5Gc, and Sd(a) that are recognized by human

antibodies, which activate certain mechanisms leading to blood coagulation [58]. Since genetic modification of pig islets have not become perfected, many countries are hesitant to approve the use of porcine islets in clinical applications to humans as it may lead to further complications. Third, in comparison to adult porcine islets, NPIs do not respond to glucose as well and are less abundant in supply. In other words, they are less sensitive to glucose stimulated insulin secretion (GSIS) compared to their adult counterparts [59]. Another hesitation of using porcine islets as a source of transplantation in humans is due to the fear of transmitting porcine endogenous retrovirus (PERV) [58, 79, 113]. Although studies have shown that PERV can possibly infect humans, there has not been any reports of PERV transmission from porcine to humans [79]. Niu et al. [113] have demonstrated the ability to deactivate PERVs in porcine cell lines using CRISPR/Cas9 via somatic cell nuclear transfer. More specifically, using two CRISPR guide RNAs (gRNAs), the researchers were able to deactivate all 62 copies of PERV in porcine somatic cell lines [113]. This ground-breaking approach has brought porcine xenotransplantation one step closer to regulated clinical application. In 2014, researchers conducted the first clinical xenotransplantation trial in New Zealand. The goal of this study was to identify the microbiological safety of alginate-encapsulated NPIs in human patients. The researchers screened for molecular markers of pig microorganisms in human patients up to 52 weeks post-transplant and found no evidence of PERV or other porcine microorganisms [163]. A follow-up of this clinical trial confirmed the absence of PERV and other porcine microorganisms five years post-transplant [164]. Although neonatal porcine islets have great potential for large-scale clinical translation, they are in some ways inferior to adult porcine islet sources.

Adult porcine islet (APIs) sources may possibly allow for optimal clinical translation, due to their high islet yield upon isolation where they compose of >70% β-cells after culture. This is

far higher than the yield for neonatal (25% β-cells after culture) and fetal (10% β-cells after culture) [115]. In addition, APIs have been demonstrated to secrete a greater amount of insulin in response to glucose compared to NPIs [59]. The histology of adult pig pancreas' show intact capsules and vasculature with few insulin positive cells outside of islet cells [115]. Another advantage of using APIs is the low number of adult pigs necessary to treat patients. It is estimated that islets from around 10 adult pigs are necessary to treat an average 70 kg patient [116]. One disadvantage of APIs is that they are less resistant to ischemia compared to NPIs [59]. Also, it is more costly to raise and maintain adult pigs in a controlled environment, and the longer time frame may increase the probability of transmission of zoonotic disease [79, 115].

Since porcine islets provide a diverse pool of islets from living donors and are available upon demand, this can potentially eliminate the problem of the shortage of human islet donors. Genetic modification of porcine islets allows for a complete elimination of PERV as well as to eliminate the surface antigens on the islet cells and may pave the way for porcine islet sources to be better translated into clinical practice.

CRISPR/Cas9 can be used to generate genetically modified pigs. This technique utilizes gRNA and Cas9 complex to recognize, unwind, and cleave the appropriate gene [66]. This results in a site-directed DNA double strand break (DSB) that leads to a frameshift mutation and the knockout of a functional gene [204, 205]. Other methods of generating modified porcine islets include using somatic cell nuclear transfer (SCNT) of genetically modified donor cells, and the generation of pronuclear DNA through microinjections of zygotes [204, 206]. Using these methods, researchers can knockout a variety of genes. This includes knockout out of α-Gal, Neu5Gc, and Sd(a) to reduce the severity of the IBMIR upon intraportal islet infusion [204]. Further, since human T-cell receptors can potentially bind to swine leukocyte antigen (SLA)

complexes (which are the homologs to human HLAs) present on the surface of porcine cells, a T-cell mediated immune response may occur to reduce the long-term survival of the islet mass [204, 207]. Therefore, researchers can also knockout genes responsible for the expression of SLA class I and II to possibly reduce the T-cell response [207, 208].

Xenogeneic sources may enable us to overcome many of the disadvantages of using human islet sources; however, further research and Health Canada regulatory approval is needed before large-scale clinical translation. In considering alternative β-cell sources, it is imperative to explore the potential of stem cells as possible sources for islet transplantation.

1-9.2 Stem Cell Sources for Insulin Producing Cells:

The drawbacks of islet transplant provide researchers with the incentive to determine alternative cell sources. One such approach is stem cell transplantation, which has been initiated in different research facilities around the globe. Stem cells can be classified in different ways, either according to their differentiation or their origin. Based on their differentiation, stem cells can be categorized into five broad categories, namely totipotent, pluripotent, multipotent, unipotent, and oligopotent. Based on their origin, stem cells can be derived from embryonic, fetal, infant, or adult sources. β-cells can be generated through four general approaches: 1) differentiating human pluripotent stem cells (hPSCs), which includes both embryonic (hESCs) and induced pluripotent stem cells (hiPSCs); 2) transmodification of other cell types (ie., hepatic, splenic, or acinar); 3) modification of pancreatic progenitor cells; and 4) proliferation of existing β-cells [165]. However, in this article only the first approach is being considered in detail.

Islet-like-β-cells that are derived from human embryonic stem cells (hESCs) and/or their adult equivalents, human-induced pluripotent stem cells (hiPSCs), have shown the potential to be used as an alternate therapy for islet transplantation [22]. Embryonic stem cells are pluripotent

undifferentiated cells derived from the inner layer of the blastocyst which is the early mammalian embryo that implants into the uterus [83]. hESCs have the capacity to proliferate indefinitely and renew themselves and are able to differentiate into multiple types of adult cells *in vitro* [125]. hiPSCs resemble hESCs in their pluripotency but are derived from adult somatic cells and are then induced to become stem cells. These pluripotent stem cells of both adult and embryonic origin can then be differentiated to become any type of somatic and germ cells, excluding placental cells.

In 1998, Thomson et al. [118] were the first to isolate embryonic stem cells from human embryos and to open the doors for cell replacement therapies [144]. However, the history of embryonic stem cell research dates farther back to 1981 when the first murine embryonic stem cells were isolated [119]. Though embryonic stem cells have been in use for many years, adult stem cells (hiPSCs) have not been used for more than 20 years [13]. In comparison, both hESCs and hiPSCs have a similar capacity to proliferate and differentiate into the vast array of somatic and germ cell lines. Many studies have also confirmed the ability of hESCs and hiPSCs to differentiate into insulin producing cells (IPCs) and stem-cell derived β-cells (SC-β) [80, 118, 120]. Yet, researchers have not been successful in mimicking the true nature of real β-cells, as these cells are missing key markers of mature β-cells. Recently, Rezania et al. [120] were able to differentiate hiPSCs into functional stem cell-derived islets (SC-βs) that expressed many of the key markers in mature β-cells, including MAFA, PDX1/NKX6-1 and INS [121]. Further, Nostro et al. [122] demonstrated the differentiation of hiPSCs to pancreatic progenitors with a high expression of NKX6-1$^+$ by using a combination of epidermal growth factor (EGF) and nicotinamide. Also, Melton and colleagues [128, 129] created a six-step protocol to differentiate hESCs to mature β-cells [13]. One important issue that arises when using hESCs during clinical translation is the ethical consideration and considering the religious backgrounds of patients

undergoing such a treatment. Since hESCs are derived from human embryos during the blastocyst stage, this should be taken into account, and the patient's consent should be acquired before undergoing such treatments. Perhaps using hiPSCs, derived from reprogrammed somatic cells, would be more reasonable when considering the religious background of patients. Further, the risk of teratogenicity should be considered when using stem cell-derived islets [13]. One other issue that should be considered when using hiPSC and/or hESC-derived pancreatic progenitors in clinical practice is the effect of the *in vivo* environment in promoting islet cell differentiation. Of particular note is that the regulation of transplanted cells in response to the *in vivo* environment has not been well documented [121]. A recent study by Maxwell et al. [209] displayed the function and maturation of transplanted hESC-derived SC-βs *in vivo* using different transplantation parameters. The group demonstrated that transplanting 2 million, but not 0.75 million cells was able to reverse streptozotocin-induced diabetes in immunodeficient mice. The authors also found that the location of transplantation resulted in different outcomes, where cells transplanted under the kidney capsule resulted in greater serum C-peptide content compared to cells transplanted in subcutaneous or intramuscular locations [209]. While this study displays the functional maturation of SC-βs *in vivo*, it is limited in using only male mice [209]. Future studies should aim to demonstrate the feasibility, functionality, and maturation of SC-βs under a range of species of different sexes, including mice, rats, and non-human primates.

Recently, clinical trials conducted by Viacyte (NCT02239354 and NCT03163511) demonstrate the capacity of hESCs to mature in an *in vivo* environment [213, 214]. Although the first clinical trial did not achieve the expected results due to insufficient engraftment [213], results from the second clinical trial utilizing VC-02 demonstrated positive results early in the trial [214]. Results from the latter trial showed that 35.3% of patients displayed positive C-

peptide stimulation at 6-months post-transplantaiton [214, 215]. In other clinical trials conducted by Vertex (NCT04786262), transplanted stem cells displayed stimulated C-peptide levels in patients, also demonstrating early positive results [216].

It should be noted that hiPSCs can be extracted from either autologous or allogeneic sources, whereas hESCs are only derived from an allogeneic source. For many years, researchers have been comparing the usage of autologous and allogeneic stem-cell derived islets in terms of their advantages and disadvantages. The next section will address the risks and benefits associated with the usage of SC-βs from either source.

1.9.2.1 Allogeneic Stem Cell-Derived Islets:

Allogeneic stem cell transplantation involves the extraction of stem cells from a healthy donor, other than the self. Donors may include siblings, family members, or unrelated donors [123]. Due to the existence of multiple alleles for each polymorphic HLA gene, the possibility of obtaining an exact match between donors and recipients is very low [22]. Thus, transplanting of allogeneic stem cell sources into the recipient can potentially result in an allo and auto-immune response to the foreign cells [22, 13]. For this reason, immunosuppressive therapy is required for a prolonged period to decrease the chance of rejection.

In one retrospective analysis, the authors demonstrated that the existence of donor-recipient HLA mismatch correlated with a longer duration of immunosuppressive therapy to treat chronic graft-vs-host disease (GVHD) [210]. In another study investigating unrelated transplant patients, it was found that patients who received HLA mismatched tissues used immunosuppressive medication for a longer period compared to those who received HLA matched tissues [211]. It may be beneficial for transplant recipients to receive a close HLA match as possible, despite having

to take immunosuppressive medications. One potential solution to increase the probability of matching HLA-homozygous cell lines between donors and transplant recipients would be to use a global stem cell bank. However, even with the US bone marrow registry with more than four million donors, there is only 50-60% match between HLA-A and HLA-B loci. An alternative method would be to use genetic engineering to create universal donor cells (UDCs) [22]. Such an approach would potentially allow for limited allogeneic stem cell donors to match a wide range of recipients. Riolobos et al. [22] have developed a method of creating UDCs where they knock out critical transcription factors involved in expressing MHC class I and II surface antigens on hESCs. Further, through the use of CRISPR/Cas9 genetic editing, researchers can create hESC or hiPSCs with reduced immunogenicity and greater functional capacity to secrete insulin [124]. Recently, Han et al. [189] were able to develop low immunogenicity SC-βs, termed hypoimmunogenic SC-βs, via CRISPR/Cas9 genome editing by deleting HLA-A/-B/-C genes and preventing the expression of HLA class II. Further, the researchers were able to maintain the expression of HLA class Ib molecules, HLA-E and HLA-G, which are required to maintain NK cell tolerance to the cells [189]. These cells, termed hypoimmunogenic SC-βs could possibly lower the required dose of immunosuppressive medications upon transplantation.

The benefit of using allogeneic hESC-derived cells is that they may be less immunogenic in comparison to human cadaveric-derived islets. This is because these hESC-derived cells will generally have a lower expression of HLA markers as opposed to human islet cells [125]. However, in one study, it was found that when hESC-derived pancreatic progenitor cells were transplanted in mice, exposure to inflammatory cytokines and IFN-γ resulted in upregulation of HLA class I markers [237]. This highlights the fact that alloimmune rejection is still a possibility even when transplanting lower immunogenicity cell products [125]. Thus, strategies must be

employed to decrease the allogeneic response. One possibility is to induce tolerance by using a cocktail of anti-CD40L and cytotoxic T-lymphocyte-associated protein 4 immunoglobulin (CTLA4-Ig) [125, 126]. Szot et al. [126] were able to successfully demonstrate that humanized (immunocompetent) mouse models transplanted with xenogeneic hESC-derived cells prevented rejection of grafts when a combination of anti-CD40L and CTLA-4Ig were used. Another method that could be used to decrease the alloimmune response in humans upon allogeneic stem cell transplant would be to ensure the matching of HLA haplotypes, or the creation of hypoimmunogenic cell lines, through the methods discussed previously [22, 189].

An important component in using allogeneic stem cell-derived islets is to ensure that the SC-βs are similar in function to mature β-cells. In other words, SC-βs should theoretically contain key biological markers of mature β-cells including, MAFA, NEUROD1, and PDX1/NKX6-1, and should have the functional features of mature β-cells such as GSIS and C-peptide secretion of comparable frequency, duration and intensity [121].

Though allogeneic stem cell-derived islets serve as a possible treatment option for the treatment of diabetes, autologous stem cell-derived islets are another readily available avenue of treatment.

1-9.2.2 Autologous Stem Cell-Derived Islets:

Autologous stem cell transplantation involves the extraction of stem cells from the recipient's own body, providing an exact HLA-match and thus reducing the alloimmune response. However, autologous transplantation of cells could potentially lead to rejection of the graft due to a persistent autoimmune response [22].

The use of autologous stem cell sources could overcome the alloimmune response from allogeneic sources. Riolobos et al. [22] demonstrated how the use of autologous hiPSCs could overcome the immunological barrier inflicted upon by allogeneic HLA types. However, the autoimmune response would still be present and therefore immunosuppressive medications would be necessary to decrease the chance of graft rejection.

Millman et al. [80] used an approach to generate autologous hiPSC-derived SC-β cells from the skin fibroblasts of T1D and non-diabetic patients (ND). β-cells derived from both populations showed no difference in terms of *in vitro* and *in vivo* function and gene expression [127]. This has significant implications; it confirms that hiPSC-derived β-cells from an autologous diabetic donor should theoretically be no different than non-diabetic donor. Further, this method could potentially provide an unlimited supply of cells, resolving the challenge of the need for multiple cadaveric donors. Of particular note is that this method may not be feasible for those with genetic mutations as proper stem cell function could be compromised [22].

As with allogeneic stem cells, autologous stem cells could also benefit from genetic modification using CRISPR/Cas9 [66]. In this context, somatic cells from the patient can be harvested and differentiated to hiPSCs, and subsequently targeted using gRNA/Cas9 complex to correct mutations, or knockout transcriptional regulators or other cell surface receptors [66]. Further, the creation of different cell lines through CRISPR/Cas9 gene editing can promote the development and testing of more feasible immunosuppressive drugs and assist in discovering accurate drug delivery methods [85].

It should also be noted that the use of autologous hiPSCs is not limited to treating only T1D. Autologous hiPSCs can be derived from a diverse population of diabetes patients including T2D, MODY, T1D, Wolfram syndrome and cystic-fibrosis related diabetes [127]. The use of

autologous and allogeneic stem cell-derived islets along with CRISPR/Cas9 gene editing has tremendous potential that could revolutionize diabetic treatment in the coming years.

For islet transplantation to potentially achieve life-long insulin independence, it is critical that researchers evaluate the usage of alternative β-cell sources in an established model. Animal studies have been instrumental in allowing researchers to conduct transplantation studies, but their usage in islet transplantation rejection studies using human immune systems are still in their infancy.

1-10 MOUSE MODELS TO STUDY GRAFT REJECTION:

There are ethical restraints in using humans as subjects for studies of islet graft rejection. Therefore, researchers have explored the use of animal models to study the mechanisms of graft rejection. Mouse models of allogeneic rejection have allowed the development of new immunosuppressive medications for clinical use. However, the immunological disparities between murine and human immune systems have made it difficult to translate animal research to clinical settings [178, 179]. One key difference between the human and mouse immune systems is the presence of reactive memory T-cells in humans may be absent in naïve mice [185]. In addition, mice lack functional TLR10 which are present in humans, and express genomes that are absent in humans such as TLR11, TLR12, and TLR13 [179, 186]. There are also differences in immune system composition between mice and humans. Human blood is neutrophil rich, with 50-70% neutrophils and 30-50% lymphocytes, whereas mice contain a much greater lymphocyte content of 75-90% and lower number of neutrophils (10-25%) [232]. Further, the difference in immunoglobin activation pathways between mice and humans demonstrates the difference in species-specific acquired immunity [232, 256]. T-cell

differentiation into Th1 is also suggested to occur through alternate stimulation pathways of different cytokines in mice and humans [256, 257]. These differences highlighted the need to develop a humanized mouse model that can realistically mimic the human immune system [178, 179, 185]. Humanized mice have been well studied over the last 30 years with the purpose of being engrafted by functioning adaptive and innate human immune systems [179]. Previous mouse models that recapitulated the human immune system were limited in their use due to the inevitable development of GVHD. These models allowed only a small timeframe to conduct immunological studies before the development of GVHD. Therefore, it has been of critical importance to develop a new mouse model that can be transplanted with a variety of β-cell sources and can be reconstituted with an authentic human immune system without developing GVHD [178, 179]. This model will allow a prolonged time frame to conduct immunological studies that were otherwise not possible.

The most common animals used in immunological and transplant studies include mice deficient in T- and B-lymphocytes either through the severe combined immunodeficiency (SCID) mutation or through the knockout of recombination-activating genes (RAG) [180]. A breakthrough occurred in the development of humanized mice when it was discovered that mutations in the IL-2 receptor common gamma chain (*IL2rg^{null}*) inhibit murine adaptive and innate immunity as well as NK cell development through defective cytokine signalling pathways [178, 179]. Under normal circumstances, the IL-2 receptor common gamma chain is responsible for high affinity receptor signalling for cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Therefore, mutating the IL-2 receptor common gamma chain eliminates cytokine signalling and induces a severely deficient strain of mice [180]. Crossing this mutation with the SCID or RAG mutation allowed for a wide array of scientific research. It has been noted that crossing the

IL-2 receptor common gamma chain and SCID mutation eliminates the "leakiness" that is present in SCID mice [180]. This leakiness is characterized by clonal expansion of leftover murine T- and B-cells that increases with age [181, 182]. This indicates that mice containing a leaky immune system are not truly immunodeficient and may not be suitable candidates to reconstitute with a human immune system. SCID mice that are deficient in the receptor common gamma chain are present on many backgrounds of mice, including Balb/c, C57BL/6, and non-obese diabetic (NOD) strains [183]. The NOD strain contains further immune tolerance as they have defective macrophage and dendritic cell function and reduced activity of the complement system [183, 184, 241]. These NOD mice that contain both the SCID mutation and IL2rg^{null} knockout are termed NSG (NOD SCID IL2rg^{null}) mice for short. These NSG mice inherently express murine MHC on their tissues and would make them an unsuitable host for engraftment of human PBMCs. Engrafting these mice with PBMCs will result in the xeno-MHC mismatch and the inevitable development of GVHD through CD8$^+$ T-cell mediated rejection [183]. To combat this, it was necessary to create a knockout of murine MHC class I and II (MHC I/II DKO) that would allow the engraftment of these mice with PBMCs without the development of GVHD. Recently, The Jackson Laboratory has been able to develop this combination of NSG-MHC-I/II DKO mice (also known as NSG-$(K^BD^B)^{null}$ $(IA)^{null}$) [258]. Preliminary studies have utilized these mice transplanted with human islets in the spleen and reconstituted them with PBMCs to determine graft rejection [184]. A study conducted by Brehm et al. [184] has shown that a majority of these mice survived up to 125 days without developing GVHD and had lower C-peptide levels 6-weeks post-reconstitution. Other preliminary studies have utilized the same approach to indicate graft rejection in human islet transplanted mice, however the use of the same NSG strain was not feasible at the time [178]. Mice used in previous studies were the

NOD.cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ, which did not contain the MHC class I and class II knockout, thus studies utilizing alternative sources of β-cells, especially hypoimmunogenic stem cell source, were not possible at the time as they require an extended timeframe to conduct immunological rejection studies. In one review, Verhoeff et al. [256] suggest that the creation of an ideal humanized mouse model with the lifespan of one year or greater could allow researchers to evaluate the survival of autologous inducible pluripotent stem cells. In this context, an extended timeframe is required to identify the possibility of recurrent autoimmunity [256]. Although this has not yet been achieved, continued advances in the development of humanized mouse models adapted for the field of islet transplantation will broaden the understanding of islet graft rejection and autoimmunity.

With the development of the new NSG-MHC-I/II DKO mice strain, it is now possible to investigate the rejection and kinetic profile of mice transplanted stem cell-derived β-cells and porcine islets in long-term rejection studies, in the context of a human immune system.

1-11 OBJECTIVES, OUTLINE, AND HYPOTHESES:

Objectives and Outline:

The primary objectives of this thesis are two-fold: 1) to demonstrate the feasibility of transplanting HIs, NPIs, and SC-βs in NSG-MHC I/II DKO immunodeficient mice, and 2) to display islet graft rejection post-reconstitution in these mice either through a reversal to the hyperglycemic state, or through the absence of stimulated insulin secretion post-reconstitution, in the absence of GVHD. To achieve these outcomes, the native immune system in NSG-MHC I/II DKO mice will first need to be investigated. Upon confirmation for the absence of a functional murine immune system, transplantation and reconstitution studies will be conducted. HI and SC-

βs will be transplanted in diabetic mice to reverse hyperglycemia, and NPIs will be transplanted in naïve, non-diabetic mice. HI and SC-β transplanted mice that achieve euglycemia will be reconstituted with a single intraperitoneal injection of PBMCs of determined doses, and subsequently monitored for the reversal to hyperglycemia, indicating islet graft rejection. NPIs will be transplanted in non-diabetic mice and the stimulated porcine insulin secretion will be determined at 4-weeks post-transplantation, followed by intraperitoneal reconstitution with determined PBMC doses. These mice will then be evaluated in terms of stimulated porcine insulin secretion at 2-, 4-, 5-, and 6-weeks post-reconstitution to determine islet rejection, indicated by functional loss of stimulated insulin secretion. To further demonstrate the feasibility of this model to conduct long-term islet graft rejection studies, the weight of NPI transplanted mice will be measured post-reconstitution and evaluated to detect the possibility of GVHD development. In all reconstituted mice, co-localization studies using immunofluorescence staining will be conducted to determine the infiltration and co-localization of human T-cells in relation to insulin or chromogranin A positive cells.

The second section of this thesis (Appendix A) investigates the cell composition of SC-β and the effect on transplantation outcomes in B6.Rag KO and NSG-MHC I/II DKO mice. Specifically, mice transplanted with SC-β <30% and SC-β ≥30% positive cells co-expressing NKX6.1 and C-peptide will be evaluated in terms of euglycemic outcomes, stimulated human insulin secretion overtime to indicate *in vivo* maturation of SC-βs, and the glucose clearance profiles following a glucose challenge test at 20-weeks post-transplant. To achieve these outcomes, diabetic B6.Rag KO and NSG-MHC I/II DKO mice will be transplanted with either SC-β <30% or SC-β ≥30% positive cells and monitored for the presence of euglycemia. At 8-, 12-, and 20-weeks post-transplantation, stimulated human insulin will be evaluated following an

IPGTT to determine *in vivo* maturation of SC-βs. At 20-weeks post-transplantation, the glucose clearance profiles will be determined following a glucose challenge test in B6.Rag mice only.

Hypotheses:

The key hypotheses in this thesis are four-fold. First, we hypothesize that NSG-MHC I/II mice will support the survival of the transplanted islets and display graft functionality, indicated either by a reversal to the euglycemic state (if diabetic) or through stimulated porcine insulin secretion (if non-diabetic) post-transplantation. Second, we hypothesize that NSG-MHC I/II DKO mice will display graft rejection post-reconstitution either by a reversal to the hyperglycemic state (if previously diabetic) or through a decline and ultimately, an absence in stimulated porcine insulin secretion (if non-diabetic). Third, we hypothesize that reconstituted mice will not display GVHD as indicated through a decrease in weight values by more than 20% from baseline during the entirety of the experiment. Lastly, we hypothesize that the infiltration and co-localization of human T-cells in relation to insulin or chromogranin A will be observed in the graft region.

Our hypotheses for our secondary experiment in Appendix A will be two-fold. First, we hypothesize that mice of either strain transplanted with SC-β ≥30% will display euglycemia to a greater degree than mice of either strain transplanted with SC-β <30%. Second, we hypothesize that B6.Rag KO mice transplanted with SC-β ≥30% will display significantly better glucose clearance during a glucose challenge test, compared to B6.Rag KO mice transplanted with SC-β <30%.

1-12 SUMMARY:

Diabetes mellitus (DM) is a metabolic disease that has affected the lives of over 400 million people as of 2014 [75]. Due to an alarming rate of increase in the prevalence of global diabetes, the need for an inclusive approach to combat this matter is of pressing concern. Nearly one hundred years have passed since the ground-breaking discovery of insulin by Banting, Best, Macleod, and Collip, which has not only transformed the treatment options available for individuals with diabetes but also expanded research in search of new treatments [2]. The improvements in surgical techniques, along with the use of more potent immunosuppressive regimens have not only decreased the morbidity rates in pancreatic transplant recipients but has also improved sustained insulin independence rates of islet transplantation to levels comparable to pancreatic transplant. The innovative techniques used in the Edmonton protocol was another landmark case in history of diabetes treatments, which has revived interest in finding a treatment for diabetes. Though there remain many drawbacks in the current clinical approach in islet transplantation, advancements in research have started to address the methodological limitations of this approach. Still, researchers are left with critical issues to address; namely the lack of revascularization in islets upon transplant, the need for multiple cadaveric donors, and the need for chronic, systemic immunosuppressive therapy post-transplant.

Researchers have currently focused their attention in discovering alternative sources of β-cells as potential means in the treatment of T1D. Xenogeneic sources, namely porcine islets have been well examined and could potentially pose as one alternative islet source. Stem cell-derived islets have gained considerable attention throughout the past 20 years and show promise in translating research on the subject into clinical practice in the coming years. As the race for exploiting the next alternative islet source advances, it is equally as important to test these islet

sources in an established mouse model with a human immune system. Such a feat will allow future researchers to further adapt the mouse model to test novel immunosuppressive medications and innovative approaches to delay graft rejection in the context of a human immune system. Such advances in therapeutic treatment options may ultimately decrease the negative consequences of current immunosuppressive regimens and increase the long-term insulin independence rates.

Though diabetes remains a critical global health issue, progressions in good research practices have systematized and accelerated the race to find more feasible treatment options. There is currently no estimated timeline in discovering a definitive treatment option for diabetes that offer patients lifelong, sustained insulin independence, but the recent breakthroughs in science and technology offer hope that it is not far.

CHAPTER TWO

HUMANIZED MOUSE MODEL DEVELOPMENT TO DEMONSTRATE HUMAN, NEONATAL PORCINE, AND STEM CELL-DERIVED ISLET GRAFT REJECTION

2-1 INTRODUCTION:

Islet transplantation is a feasible option to treat patients with type 1 diabetes. However, the existence of major barriers limits this treatment option for many patients. In particular, the use of systemic immunosuppressive medications and the limited human islet source are major obstacles that must be overcome to allow for greater acceptance of islet transplantation. The use of alternative β-cell sources is an attractive substitute to conventional human islet usage. Specifically, the use of neonatal porcine islets (NPI) as well as stem cell-derived beta cells (SC-β) pose promising future treatment options. Xenotransplantation of NPIs have previously been used in clinical trials in New Zealand and Mexico [164, 217]. In non-human primate models of diabetes, NPI transplantation has shown to reverse associated hyperglycemia and delay rejection when provided with immunosuppressive agents [218]. As such, the usage of NPIs could accompany the human islet source as a near-treatment option for patients with type 1 diabetes. SC-β could pose a promising future treatment option in the long-term. Clinical trials of stem cell-derived islets from ViaCyte and Vertex are in their infancy and much more research needs to be established to bring stem cell treatments of diabetes to the forefront [219 – 222]. To bring NPI and SC-β treatment options towards widespread clinical adoption, it is imperative to evaluate the immunogenicity of these cells in the context of a human immune system.

There are major ethical limitations in using humans as recipients of experimental islet transplantation. Therefore, the next best option is to use an established mouse model that can be engrafted with a human immune system. Out of the many murine strains previously deployed, non-obese diabetic SCID-gamma (NSG) mice remain as a fundamental resource for experimental work due to their severe combined immunodeficiency (SCID) along with their absence of functional T-, B-, and Natural Killer (NK) cells [223, 224]. However, one

fundamental flaw in this original strain is the presence of the mouse major histocompatibility complex (MHC) class I and class II which limits the application of this species to become reconstituted with a human immune system. Upon reconstitution, this strain may eventually develop graft versus host disease (GVHD) which occurs when the engrafted human immune system recognizes the mouse host tissue as a foreign entity, and eventually exterminating the host [223, 179]. Consequently, this limits the timeframe to conduct long-term immunological research studies. One solution to this would be to use a specific strain of NSG mice that lack MHC class I and class II (NSG-MHC I/II DKO). As a result, future research will be able to investigate long-term graft rejection studies without the limited timeframe posed by GVHD [223, 179]. To further establish a humanized mouse model, the context of the human immune system must first be defined.

The human immune system is a multifaceted network of cells and organs that provide specific lines of defence to combat diseases [225, 226]. Although the human immune system is complex and integrated in the context of a human host, parts of this system can be transferred from a host to a recipient [227]. One commonly used method to isolate human white blood cells from red blood cells and plasma is called leukapheresis. Through the insertion of two intravenous (IV) lines along with adjustable flow rates, blood is circulated through an Apheresis machine, which separates blood into its individual components, returning the red blood cells and plasma to the body [228]. White blood cells, and specifically, peripheral blood mononuclear cells (PBMCs), are isolated, and include lymphocytes (T-, B-, and Natural killer cells), monocytes, and dendritic cells, but do not include neutrophils and eosinophils [229, 230]. For experimental purposes, these human immune cells can be transferred into murine models described above to conduct long-term immunological rejection studies.

To more closely understand how the human immune system responds to transplanted islets and to test a variety of strategies to prevent or delay T-cell mediated graft rejection, it is imperative to have an established rodent model that can be transplanted with a variety of islet sources to correct diabetes and display T-cell mediated graft rejection, analyzed through quantitative and qualitative methods. This project aims to accomplish the goals mentioned above by creating a humanized mouse model that can be transplanted with a variety of β-cell sources and display islet graft rejection upon reconstitution with a human immune system. In short, the establishment of this model will allow for the assessment of a variety of strategies to prevent or delay islet graft rejection in the future.

2-2 EXPERIMENTAL DESIGN:

2-2.1 Mice:

Six-week-old male NOD.SCID gamma ($K^bD^b)^{null}(IA^{null})$ mice (NSH-MHC I/II DKO) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used as recipients for human islet (HI) (n=25), NPI (n=24), and SC-β (n=5) transplantation. All mice were housed in a pathogen-free, climatized environment at the Health Sciences Laboratory Animal Services Facility of the University of Alberta. All animals were fed standard laboratory food and given water containing Novotrimol *ad libitum*. Animal use was in accordance with the Canadian Council on Animal Care and approved by the institutional animal ethics committee at the University of Alberta, Edmonton AB, Canada (AUP00000278, AUP00002977).

Mice used for transplantation of HIs and SC-β, and subsequent reconstitution, were made diabetic through intraperitoneal (IP) injection of streptozotocin (STZ) at 185 mg/kg (MilliporeSigma, St. Louis, MO, USA) mixed in acetate buffer (pH 4.5). Mice were confirmed to

be diabetic when the blood glucose level was ≥17.7 mmol/L for two consecutive days. Mice that did not achieve a blood glucose level of ≥17.7 mmol/L were not used for transplantation. Once diabetes was confirmed, each mouse received a LinBit (Linshin, Toronto, Canada) implanted subcutaneously. Non-fasting blood glucose and weights of mice were monitored 2-3 times per week post-transplantation and post-reconstitution with human PBMCs. Naïve, non-diabetic mice were used for transplantation of NPIs and maintained a blood glucose level of ≤11.1mmol/L. The weights of these mice were monitored 2-3 times per week prior to reconstitution.

2-2.2 Assessing the Native Immune System in NSG-MHC I/II DKO Mice:

To assess the possibility of the presence of a functional immune system (i.e., leakiness) in the NSG mice, the transplanted human islet graft of a small sample of mice were analyzed using immunohistochemistry. Immunofluorescent staining in one cohort of human islet transplanted mice that did not normalize was used to confirm the absence of the adaptive immune system in this model. In brief, the rationale was to rule out the possibility of murine-specific islet graft rejection.

Following euthanasia of mice, graft-bearing kidneys were collected and specimens for immunohistochemical analysis were immersed in optimal cutting temperature (OCT) compound (Thermo Fisher Scientific, Waltham, MA, USA) and directly stored at -80°C. Tissue sections of 5 um thickness were sliced, and slides were airdried for 5 minutes then fixed in acetone at -20°C for two minutes before being airdried again for another 5 minutes. Tissue sections were blocked with 20% normal goat serum (NGS, Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for 60 minutes. Subsequently, tissue sections were applied with either rat anti-mouse CD4 (1:200, Bio-Rad Laboratories, Hercules, CA, USA), rat anti-mouse CD8 (1:200, Bio-Rad

Laboratories), or rat anti-mouse CD68 (1:200, Bio-Rad Laboratories) antibody and incubated for 60 minutes. Following, tissue sections were incubated with goat anti-rat Alexa Fluor 594 secondary antibody (1:200, Thermo Fisher Scientific) for 60 minutes. Succeeding, tissue sections were incubated with guinea-pig anti-human insulin (1:5, Agilent, Santa Clara, CA, USA) for 60 minutes and then with goat anti-guinea-pig Alexa Fluor 488 secondary antibody (1:200, Thermo Fisher Scientific) for 60 minutes. Slides were then cover slipped and left in a dark box for visualization.

2-2.3 Preparation of Islets:

Human Islets:

Human islets were obtained from the Alberta Diabetes Institute (ADI) Islet Core, through the Human Organ Procurement and Exchange Program in Alberta. Human islets were cultured in Connaught Medical Research Laboratories (CMRL-1066, Corning-Costar Corporation, Cambridge, MA, USA) medium supplemented with bovine serum albumin (BSA, 0.5% v/v, Equitech-Bio Inc., Kerrville, TX, USA), Insulin-transferrin selenium (ITS, Corning-Costar Corporation), Glutamax (Gibco, Waltham, MA, USA), and Penicillin-Streptomycin (Lonza Bioscience, Basel, CHE) prior to receival. Afterwards, islets were aliquoted into 2000 islet equivalents (IEQ) per recipient in Eppendorf tubes for transplantation. All studies were approved by the Human Research Ethics Board at the University of Alberta (Pro00092479). A summary of the characteristics of human islets as well as the details of transplantation and reconstitution of mice are summarized below in Table 1.

Table 1: Characteristics of human islets received from 5 independent donors. Overall, 21 male NSG-MHC I/II DKO mice were transplanted with human islets, however 9 mice that became normalized were included in the reconstitution and rejection study. 7 and 2 mice received 40 and 60 million human PBMCs, respectively. All normalized mice that were reconstituted with human PBMCs displayed metabolic rejection as indicated through a return to the hyperglycemic state. Mice from cohort 2 received thawed PBMCs from donor 1. Mice from cohorts 3 and 4 received fresh PBMCs from donor 2, whereas mice from cohort 5 received thawed PBMCs from donor 2. Mice in cohort 1 were not reconstituted with PBMCs and were used for the purpose of assessing the native immune system.

Cohort #:	1	2	3	4	5
			Transplantation and Reconstitution		
Mice (n=):	n=5	n=5	n=4	n=3	n=4
Normalized:	None	5/5	¼	1/3	2/4
Reconstituted:	None	5/5	¼	1/3	2/4
PBMC Doses:	None	n=3 (40×10^6) n=2 (60×10^6)	40×10^6	40×10^6	40×10^6
PBMC Donor #:	None	1	2	2	2
Fresh/Thawed PBMCs:	NA	Thawed	Fresh	Fresh	Thawed
Rejection Ratio:	NA	5/5	1/1	1/1	2/2
			Human Islet Characteristics		
Human Islet Batch #:	R382	R409	R419	R420	R425
Donor Age (years):	45	9	70	55	36
Sex:	Male	Male	Male	Male	Female
Purity (%):	90	80	80	85	80
BMI:	29.7	18.1	31.5	23.5	25.2
Culture Time (hours):	12	64	31	26	19

Neonatal Porcine Islets:

Islets from 1–3-day-old Duroc Landrace neonatal pigs were isolated based on the methods established by Korbutt et al. in 1996 [109]. Neonatal pigs were anaesthetized and exsanguinated through abdominal aortic catheterization. The pancreas was removed and placed in Hank's Balanced Salt Solution (HBSS, MilliporeSigma). Subsequently, the pancreas was aseptically cut and digested in 1.0 mg/ml collagenase (MilliporeSigma) and filtered through 500 µm nylon screens for purification. NPIs were cultured in a 150 mm Petri dish with Hams F10 Medium (MilliporeSigma) supplemented with protease inhibitor (PI) and caspase inhibitor (CI) for 24 hours. The next day, a full media change was performed using Hams F10 Medium supplemented with PI and ROCK inhibitor. On day 3, the last full media change was performed using Dulbecco's Modified Eagle Medium nutrient mixture F12 (DMEM F12, Gibco) supplemented with nicotinamide and Exendin 4. Cells were left in culture at 37°C with 5% CO_2 and 95% oxygen until day 7 in preparation for transplantation. The islets were counted and aliquots of approximately 4000 NPIs per recipient were pipetted into Eppendorf tubes and allowed to gravity settle in the incubator until time of transplant. Animal use was in accordance with the Canadian Council on Animal Care (CCAC) and approved by the institutional animal ethics committee at the University of Alberta, Edmonton AB, Canada. A summary of the details of transplantation and reconstitution of mice are included in Table 2.

Table 2: Summary of neonatal porcine islet transplants in mice and the outcome of results. A total of 24 mice were used as recipients of transplantation and 21 mice were reconstituted. Overall, 17 mice were used as recipients of intraperitoneal glucose tolerance tests (IPGTT) and the results are analyzed at different time points. The kidney-bearing islet grafts of 21 mice were imaged to observe the infiltration and co-localization of human CD45$^+$ immune cells in relation to chromogranin A. Mice in cohorts 1 and 2 received thawed PBMCs from donor 1. Mice in cohorts 3, 4, 5 and 6 received PBMCs from donor 2. Cohorts 3 and 4 received fresh PBMCs which were immediately prepared after shipment. Thereafter, the next cohorts, 5 and 6 received thawed PBMCs.

Cohort #	1	2	3	4	5	6
Transplantation and Reconstitution						
Mice (n=):	n=4	n=5	n=5	n=5	n=3	n=2
Mortalities:	None	None	None	n=3	None	None
Reconstituted:	4/4	5/5	5/5	2/2	3/3	2/2
PBMC Doses:	n=2 (20×10^6) n=2 (40×10^6)	n=2 (20×10^6) n=3 (40×10^6)	n=2 (20×10^6) n=3 (40×10^6)	n=2 (40×10^6)	n=3 (40×10^6)	n=2 (40×10^6)
PBMC Donor #:	1	1	2	2	2	2
Fresh/Thawed PBMCs:	Thawed	Thawed	Fresh	Fresh	Thawed	Thawed

Stem cell-derived Islets:

Stem cell-derived islets (SC-β) differentiated from an induced pluripotent cell line (IPS) (University of Toronto, Toronto, Canada) were kindly provided by Dr. Nostro's lab at the University of Toronto. Cells were differentiated in culture for 21-25 days based on the protocol established by Hogrebe et al. [231]. At 19-20 days, the media was changed to a solution of MCDB-131 (Wisent Bio Products, Saint-Jean Baptist, QC, CA), supplemented with 7.5% sodium bicarbonate (Gibco), Glutamine, D-Glucose (MilliporeSigma), Zinc Sulfate (MilliporeSigma), Fatty acid free Bovine serum albumin (FAF-BSA, ProLiant Biologicals, Ankeny, IA, USA) and the cells were shipped overnight in a 50 mL conical tube containing this media. Prior to shipment, cells were assessed for markers of mature β-cell phenotype co-expressing NKX6.1 and C-peptide using flow cytometry. Upon receival, islets were gravity settled and the pellet was removed and put into a 150 mm non-coated petri dish. The media was centrifuged at 1200 rpm for two minutes to remove dead cell debris and this media was then used to culture the islets overnight at 37°C with 5% CO_2 and 95% oxygen. The following morning, approximately 5.0×10^6 cells per recipient were aliquoted into Eppendorf tubes in preparation for transplantation. All studies were approved by the Human Research Ethics Board at the University of Alberta (Pro00092479). A summary of the characteristics of SC-βs as well as the details of transplantation and reconstitution of mice are summarized below in Table 3. Further analysis regarding the cell composition of SC-β transplantation in mice is described in Appendix A.

Table 3: Characteristics of stem-cell derived islets transplanted in mice. A total of 5 mice were transplanted and 3 mice achieved normoglycemia and were reconstituted. Out of the reconstituted mice, 2 displayed rejection as indicated by hyperglycemia and were used in the analysis. The mouse that did not revert to hyperglycemia was excluded from the analysis.

Cohort #:	1	2
Transplantation and Reconstitution		
Mice (n=):	n=2	n=3
Mortalities:	None	1/3
Normalized:	1/2	2/2
Reconstituted:	1/1	2/2
PBMC Doses:	40×10^6	40×10^6
PBMC Donor #:	2	2
Fresh/Thawed PBMCs:	Fresh	Thawed
Rejection Ratio:	1/1	1/2
Stem Cell-Derived Islets Characteristics		
Stem Cell ID:	ASIL18 D24	FSIL16 D24
Shipment Date:	August 4, 2021	November 17, 2021
Receival Date:	August 5, 2021	November 18, 2021
Overnight culture:	Yes	Yes
Cell count (vial)	20.4×10^6 cells	15.0×10^6 cells
Cell count (hemocytometer):	22.27×10^6 cells	13.47×10^6 cells
Double positive NKX6.1 & C-peptide (%):	24.9	60.3

2-2.4 Islet Transplantation and Reconstitution:

Transplantation Procedure:

Mice undergoing transplantation were anaesthetized through isoflurane inhalation (1.5%) and the left flank was shaved and disinfected using 70% ethanol. The skin was swabbed three times with chlorhexidine (Thermo Fisher Scientific) using a sterile gauze. Mice were subcutaneously administered buprenorphine (0.05-0.1 mg/kg) (Western Drug Distribution Center Ltd., Edmonton, AB, CA) using a 27-guage needle and 1 mL syringe. Afterwards, the left kidney was localized through the skin of the mouse and an incision was made in the skin and muscle layer near the shaved flank area using iris scissors. The kidney was uncovered from the peritoneal cavity using a sterile cotton swab and a small incision was made in the kidney capsule (KC) using a 27-gauge needle. Aliquots of either HIs, NPIs, or SC-βs were aspirated into polyethylene (PE-90) tubing, pelleted by centrifugation, and then gently transferred under the KC with the aid of a micromanipulator syringe. Following transplantation of the islets, the KC was cauterized using a cautery pen to prevent the leakage of transplanted cells. The kidney was then placed back into the perirenal space and the incision in the muscle layer was sutured using a 5-0 Vicryl suture thickness and the skin was stapled. Mice were then placed in a warm cage and closely monitored for recovery. All transplanted mice were closely monitored for the presence of low blood glucose ($\leq$ 4 mmol/L) and subsequently administered 100-200 µL of D-glucose via IP injection. The LinBits of mice transplanted with HIs were removed at the time of transplantation. LinBits given to mice transplanted with SC-βs were replaced during the time of transplantation and subsequently removed at 4-weeks post-transplant.

Peripheral Blood Mononuclear Cell Preparation:

Human peripheral blood Leukopaks were acquired from StemCell Technologies (StemCell Technologies, Vancouver, BC, CA) from two independent female donors. Cells were received in an intravenous (IV) bag, and donor characteristics were provided by StemCell Technologies. Leukopaks were processed approximately one year apart and placed in cryogenic storage dewars upon arrival, if not immediately used for reconstitution. Characteristics of PBMCs are summarized in Table 4 below. The donors were both female with differences in ethnicity, age, and blood type (Table 4). No overt differences in function were observed in mice reconstituted with either donor. PBMCs from each donor were separately stored and not mixed during the entirety of the experiments.

Cells were injected into mice either fresh (upon delivery) or thawed. Cells from single donors were thawed (if frozen) in a 37°C water bath and pooled together in one 15 mL conical tube and thoroughly mixed. 100 µL of cells were removed and diluted to 40X using a mixture of 3% acetic acid with methylene blue (StemCell Technologies), or trypan blue (MilliporeSigma) and counted using a hemocytometer. Pooled cells were washed twice with HBSS and centrifuged at 1200 rpm to remove red blood cells and debris in the supernatant. Once the supernatant was removed from final wash, the cells were re-suspended in warm Hams F10 Medium and aliquoted into Eppendorf tubes of either approximately 20, 40, or 60 million cells. A 25-guage needle was used to inject transplanted mice via IP administration.

Table 4: Peripheral blood mononuclear cells acquired through StemCell Technologies and provided by two donors. Approximately 1.00×10^9 more cells were acquired from donor two. Both donors were female and non-smokers, with differences in age, ethnicity, and blood type. The viability of cells from both donors were 99%.

Donor #	1	2
Leukopak ID	#110040436	#888663859
Cell processing date	November 17, 2020	November 30, 2021
Age	53	29
Sex	Female	Female
Weight	83kg	73kg
Height	168cm	163cm
Ethnicity	Caucasian	Hispanic
Smoker	No	No
Anticoagulant	ACDA	ACDA
Viral testing	Negative for HIV-1, -2, HEP-B, -C (November 11, 2020)	Negative for HIV-1, -2, HEP-B, -C (November 22, 2021)
Blood type	A+	O+
Viability	99%	99%
Cell count	1.61×10^9 cells	2.61×10^9 cells

Reconstitution:

HI transplanted mice that achieved euglycemia within 4 weeks (n=9) were injected with a single IP injection of either 40 or 60 million human PBMCs and were monitored for rejection as indicated by hyperglycemia. Naïve, non-diabetic mice transplanted with NPIs underwent a 4-week IPGTT post-transplantation to obtain stimulated porcine insulin baseline values to compare to post-reconstitution values. At 6-weeks post-transplant, NPI transplanted mice (n=21) were reconstituted through a single IP injection of either 20 or 40 million PBMCs. Mice transplanted with SC-βs that achieved euglycemia at 16 weeks or sooner (n=3) were reconstituted through a single IP injection of 40 million human PBMCs and subsequently monitored for rejection, indicated by a return to hyperglycemia.

2-2.5 Metabolic Follow-up:

Blood Glucose and Weight Measurements:

Non-fasting blood glucose (BG) measurements of mice transplanted with HIs and SC-βs were obtained from the tail vein (OneTouch UltraMini glucose meter). Mice were considered euglycemic when the blood glucose level was ≤11.1 mmol/L for one consecutive week. Weight values of mice transplanted with NPIs were obtained once weekly prior to reconstitution. Following the reconstitution of mice with human PBMCs, mice transplanted with HIs and SC-βs were considered diabetic when the blood glucose level was ≥17.7 mmol/L, indicating islet graft-specific rejection. Rejection in NPI transplanted mice was determined using stimulated porcine secretion values post-reconstitution compared to pre-reconstitution values. Rejection in NPI transplanted and reconstituted mice was defined as stimulated porcine secretion values at or near

the lowest limit of detection of the ELISA standard curve (2.78 mmol/L) for both basal (time 0) and stimulated (time 60) values.

Stimulated Graft Insulin Secretion:

An intraperitoneal glucose tolerance test (IPGTT) is a procedure used to assess glucose tolerance in transplanted recipient mice. To evaluate the degree of function and rejection in naïve mice transplanted with NPIs, an IPGTT was conducted at 4-weeks post-transplantation and subsequently at 2-, 4-, 5-, and 6-weeks post-reconstitution. In mice transplanted with SC-βs, an IPGTT was conducted at 8- and 12-weeks post-transplantation to assess the *in vivo* functionality of islets. An extensive analysis of the cell composition of SC-βs and the outcome of transplantation results can be found in Appendix A. After a 12-hour fast, blood samples were obtained from the tail vein at 0 minutes. Following, D-glucose (3 mg/g) was administered IP using a 25- or 27-gauge needle, and blood was again collected from the tail vein at 60 minutes. Concurrently, blood glucose measurements were taken from the tail vein at 0 and 60 minutes. Immediately, samples were placed on ice and centrifuged at 10,000 rpm at 4°C for 10 minutes.

Serum samples were collected to detect the presence of graft-specific porcine insulin (NPI transplant) or human insulin (SC-β transplant), and subsequently stored in -20°C. Fasting (0 minute) and stimulated (60 minute) porcine and human insulin levels were measured using ALPCO enzyme-linked immunosorbent assay (ELISA) (ALPCO, Salem, NH, USA). This assay detects human insulin at 100% and cross-reacts with porcine insulin at 175% but does not cross-react with mouse or rat insulin (0%).

2-2.6 Graft Characterization:

To detect the presence and co-localization of human immune cell infiltration in islet graft-bearing kidney grafts, slides were co-stained with rabbit anti-human CD45 (1:350, ABCAM, Cambridge, UK), and guinea-pig anti-human insulin (1:5) or anti-chromogranin A (1:100, Novus Biologicals, Centennial, CO, USA). In mice transplanted with HI and SC-βs, human insulin was stained in addition to human $CD45^+$ immune cells. In mice transplanted with NPIs, chromogranin A was used as an alternative, in addition to human CD45 staining.

Graft-bearing kidneys were collected once islet graft-specific rejection was determined. Specimens for immunohistochemical analysis were fixed at 10% paraformaldehyde (BDH Laboratory Supplies) and then embedded in paraffin. Tissue sections of 5 μm thickness were sliced. Immunofluorescent stained slides were cover slipped using ProLong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) and left in a dark box until visualized using the Zeiss COLIBRI Widefield Florescence Microscope (ZEISS, Oberkochen, DEU). For slides stained using ABC-DAB, slides were visualized using the Nikon ECLIPSE TS2 inverted microscope (Nikon, Melville, NY, USA).

Immunohistochemistry and Immunofluorescence:

Tissue sections used for immunochemistry were rehydrated and subject to heat-mediated antigen retrieval using Tris-EDTA (pH 9.00) (if stained for CD45 or insulin) or citrate (pH 5.50) (if stained for chromogranin A). Slides were quenched in a mixture of methanol and hydrogen peroxide for 6 minutes to remove endogenous peroxidase and the quenching reaction was immediately stopped by placing the slides in water. Afterwards, slides were incubated for 60 minutes with 20% NGS. Subsequently, the slides were incubated with either guinea-pig anti-

human insulin antibody (1:5) or rabbit anti-chromogranin A antibody (1:400, ABCAM, Cambridge, UK) for 60 minutes, or with rabbit anti-human CD45 antibody (1:350, ABCAM, Cambridge, UK) overnight at 4°C. Next, an incubation was performed with either biotinylated goat anti-guinea-pig IgG secondary antibody (1:200, Thermo Fisher Scientific), or biotinylated goat anti-rabbit IgG secondary antibody (1:200, Jackson ImmunoResearch Laboratories Inc) for 60 minutes. Avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA, USA) was applied to the tissue sections and incubated for 40 minutes, followed by application of 3,3-diaminobenzidine (DAB, BioLegend, San Diego, CA, USA) to produce a brown color for positive cells. Subsequently, the reaction was immediately stopped by placing the slides in water, and the sections were counterstained with Harris' hematoxylin and eosin (H&E) and cover slipped for visualization.

Tissue sections were simultaneously analyzed for the co-localization of insulin and anti-human CD45 using immunofluorescence. After rehydration and heat-mediated antigen retrieval using Tris-EDTA, tissue sections were initially blocked with 20% NGS for 60 minutes. Following the blocking process, tissues were incubated with rabbit anti-human CD45 antibody (1:350) and incubated overnight at 4°C. The next day, the tissue sections were incubated with goat anti-rabbit Alexa Fluor 594 antibody (1:200, Thermo Fisher Scientific) for 60 minutes. Subsequently, tissue sections were incubated with guinea-pig anti-human insulin antibody (1:5) for 60 minutes, followed by goat anti-guinea pig Alexa Fluor 488 antibody (1:200) for 60 minutes.

Due to the low amount of insulin positive cells in NPIs, the co-localization of chromogranin A and anti-human CD45 using immunofluorescence was completed in NPI transplanted and reconstituted mice. Stained tissue sections first underwent heat-mediated

antigen retrieval using Tris-EDTA and blocked with 20% normal donkey serum (NDS, Jackson for ImmunoResearch Laboratories Inc) for 60 minutes. This was followed by the application of rabbit anti-human CD45 antibody (1:350) incubated overnight at 4°C. The next day, the tissue sections were incubated with donkey anti-rabbit Alexa Fluor 594 antibody (1:200, Thermo Fisher Scientific) for 60 minutes. Subsequently, tissue sections were incubated with sheep anti-chromogranin A antibody (1:100, Novus Biologicals, Centennial, CO, USA) for 60 minutes, followed by donkey anti-sheep Alexa Fluor 488 IgG antibody (1:200, Thermo Fisher Scientific) for 60 minutes.

2-2.7 Statistical Analysis:

Data are represented as mean ± standard error of the mean (SEM). Within each experimental condition, differences between groups were analyzed using one sample, two-way t-test, or a one-way ANOVA with Tukey's post-hoc test for analysis of variances for multiple comparisons in each group. The median-survival rate of human islet and stem-cell transplanted mice were analyzed using a Kaplan-Meier Log-rank (Mantel-Cox) test. All comparisons were performed using a 95% confidence interval and a p-value of $*p<0.05$ was considered statistically significant. Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, La Jolla, Ca, USA).

2-3 RESULTS:

2-3.1 Investigating the Native Immune System in NSG-MHC I/II DKO Mice:

Two mice transplanted with human islets in cohort 1 were selected at random and used to investigate the native immune system in NSG-MHC I/II DKO mice. Immunostaining of human islet bearing kidney grafts in mice confirmed the absence of murine-specific CD4$^+$ (Figure 1A, B) and CD8$^+$ T-cells (Figure 1C, D). In addition, the presence of CD68$^+$ murine macrophages were visually confirmed (Figure 1E, F), though stated to be defective in function in NSG mice, according to The Jackson Laboratory [184, 241]. The absence of murine specific CD4$^+$ and CD8$^+$ T-cells confirms that the T-cell mediated infiltration of islet grafts observed in the subsequent groups were specifically from the reconstituted human PBMCs. Leakiness in these mice was confirmed to be absent as functional murine-specific lymphocytes were not observed.
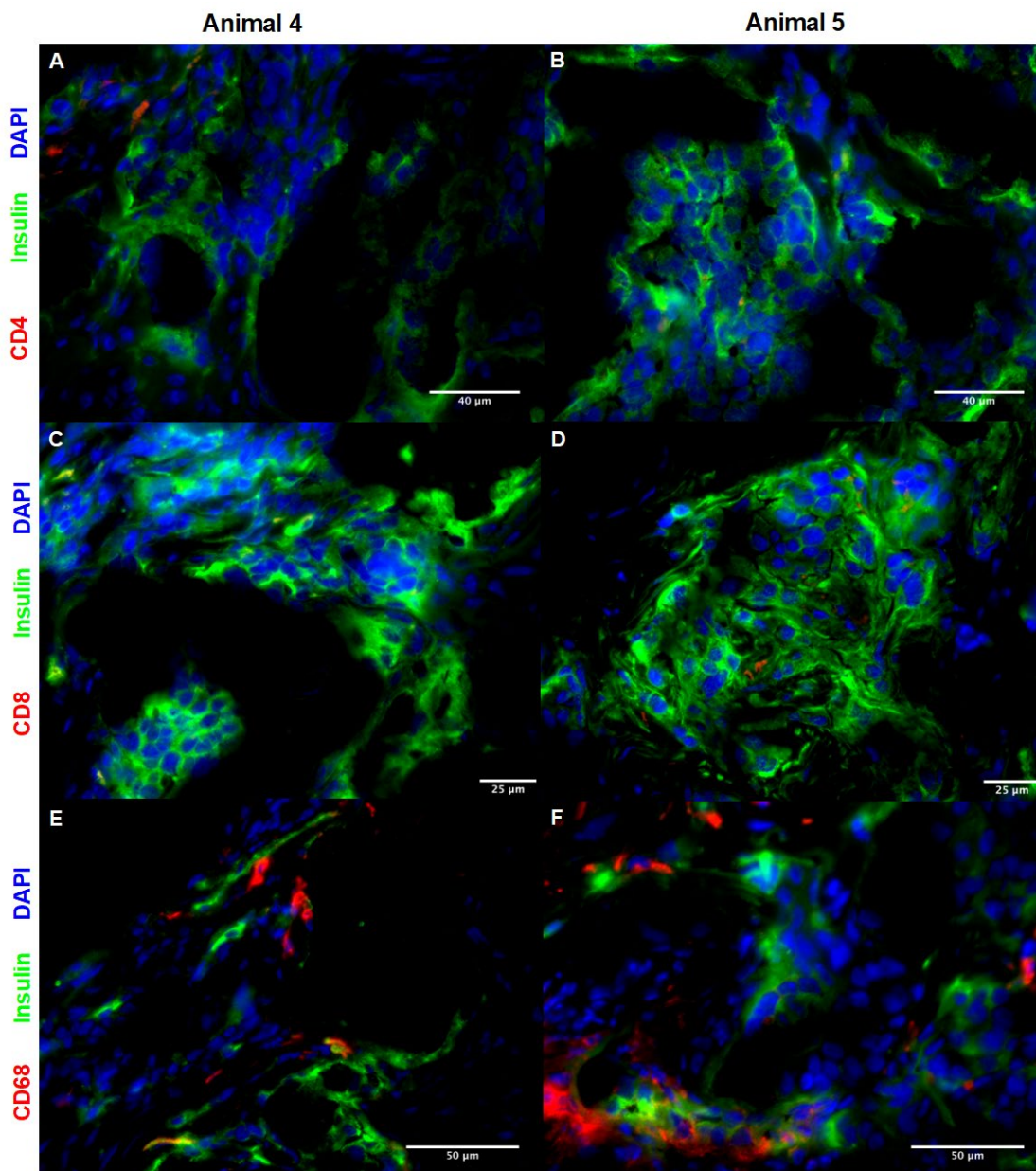
Figure 1: Immunofluorescent staining images investigating "leakiness" of a functional murine immune system in NSG-MHC I/II DKO mice. Two mice were selected at random and stained to investigate the murine immune system. In all sections, insulin staining is observed in green and staining of the nuclei with DAPI is observed in blue. **A & B)** Murine specific CD4 (red) staining was absent in the islet graft region. Red stain on image A displays background staining. **C & D)** Mouse specific CD8 (red) staining was absent. Red stain on image D displays background staining. **E & F)** Mouse specific CD68 (red) staining for macrophages are present in this model, though they are stated to be defective in function. All images are displayed at 40X magnification and mice were euthanized 45 days post-transplantation.

2-3.2 Analysis of Human Islet Transplantation and Reconstitution:

A total of 21 mice were transplanted with human islets from 5 human islet donors. 9 mice that achieved euglycemia (BG ≤11.1 mmol/L) within 4 weeks of transplantation were used for the reconstitution experiment. Human islet donors differed in sex, age, body mass index (BMI), and HLA typing (not displayed) (Table 1).

Metabolic follow-up of non-fasting blood glucose was measured on HI transplanted mice weekly. Out of the 9 mice that previously normalized and were reconstituted, 100% displayed a reversal to the hyperglycemic state indicating islet graft rejection. Mice provided with 40 million PBMCs displayed a reversal to hyperglycemia as early as 5 days and as late as 18 days post-reconstitution (Figure 2A). The 2 mice provided with 60 million PBMCs reverted to hyperglycemia at 16 days post-reconstitution (Figure 2B).

To understand if there was a difference in the timepoint of rejection in mice post-reconstitution, previously normalized mice that were given 40 million (n=7) or 60 million (n=2) PBMCs were compared based on the median timepoint of rejection using a Kaplan-Meier Log-rank (Mantel-Cox) test. A comparison of the medians showed that there was no statistically significant difference between these two groups (p>0.05). According to the results, the median rejection period of human islet transplanted mice reconstituted with 40 million PBMCs (n=7) were 10 days, compared to those reconstituted with 60 million PBMCs (n=2), which was 16 days (Figure 1C).

Immunofluorescent staining indicated the infiltration and co-localization of human CD45$^+$ immune cells in the presence of human insulin cells at 20X and 40X magnification (Figure 3A-D). This was confirmed in mice reconstituted with either 40 or 60 million PBMCs. In addition, DAB staining of consecutive sections of the graft demonstrated positive staining for

human insulin and human CD45^{+} cells in relation to the morphological features of the KC and

islet graft (not displayed).
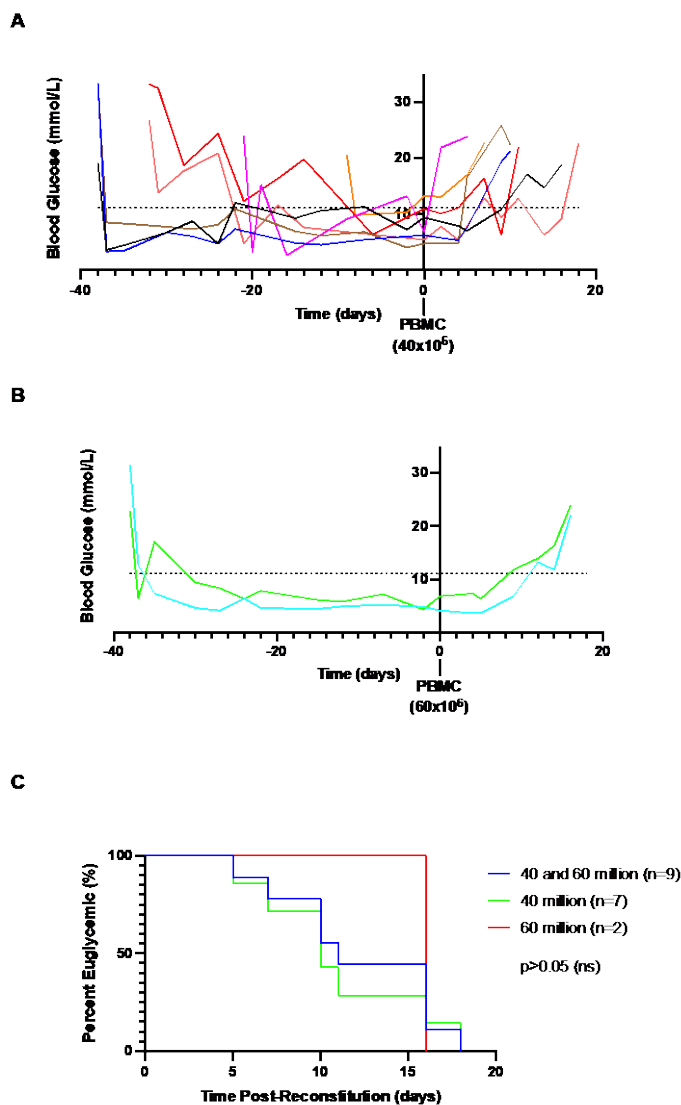
Figure 2: A) Metabolic profile of human islet transplanted mice reconstituted with 40 million human PBMCs. 7 mice were used in the reconstitution study. Mice reverted to hyperglycemia as early as 5 days and as late as 18 days post-reconstitution. **B)** Metabolic profile of human islet transplanted mice reconstituted with 60 million human PBMCs. 2 mice were reconstituted, and both mice reverted to hyperglycemia at 16 days post-reconstitution. **C)** Survival curve of human islet transplanted mice reconstituted with 40 and 60 million PBMCs. Using a Kaplan-Meier Log-rank (Mantel-Cox) test, the median survival time of mice from the two experimental conditions were compared and graphed (green and red). The mean rejection time for mice in both groups were also taken (red) to indicate the average timepoint of rejection. (C; ns $p > 0.05$).
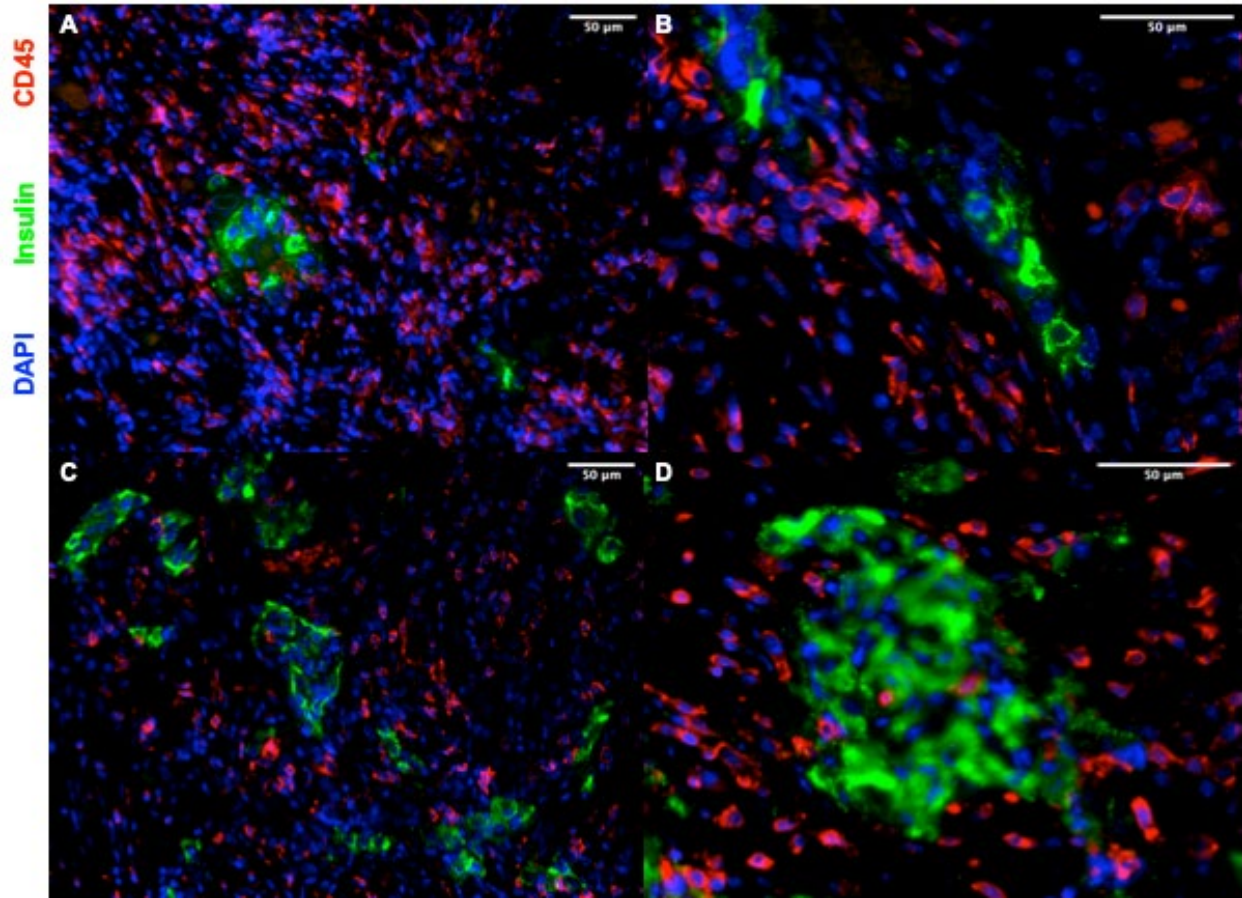
Figure 3: Immunofluorescence staining of human islet transplanted mice reconstituted with 40 million PBMCs in cohort 2 euthanized at 10 days post-reconstitution (**A & B**), or 60 million PBMCs in cohort 2 euthanized at 16 days post-reconstitution (**C & D**). Image displays the observed co-localization of human CD45$^+$ cells (red) infiltrating the human islet graft region stained with insulin (green). The nuclei of individual cells are stained with DAPI (blue). No visual differences were observed in the number of human immune cells infiltrating the graft region. Images display the graft region at 20X (A, C) and 40X (B, D) magnification.

2-3.3 Analysis of Neonatal Porcine Islet Transplantation and Reconstitution:

A total of 24 naïve, male NSG-MHC I/II DKO mice were transplanted with approximately 4000 IEQ of NPIs. Out of the transplanted groups, 21 mice survived and were reconstituted with human PBMCs. Overall, 6 of these mice were reconstituted with 20 million PBMCs, whereas 15 were reconstituted with 40 million PBMCs. A total of 21 mice were used to identify human CD45$^+$ immune cell graft infiltration. Mice in cohort 1 were administered with human PBMCs but were not used as recipients for IPGTT. Rather, mice in this cohort were euthanized at 20 and 40 days to confirm the presence and co-localization of human CD45$^+$ immune cells in the porcine islet graft region at different time points. Upon confirmation of graft infiltration in this first cohort, forthcoming groups were used to analyze the functional decline of porcine insulin release due to porcine islet rejection mediated by human T-cells.

To assess NPI graft function and subsequent rejection in mice, serum porcine insulin levels were analyzed following the administration of an IP injection of glucose at 0 minute (basal) and 60 minutes (stimulated) and the values were compared pre- and post-reconstitution. During analysis, insulin that was below the lower limit of detection (i.e., non-detectable) on the ELISA standard curve was assigned the lowest value on a standard curve (2.78 pmol/L). All values were taken to two decimal points and outlier values were removed from analysis. In addition, the stimulation index (SI) was calculated as the ratio of stimulated/basal porcine insulin secretion

A total of 17 mice were used for analysis of porcine islet graft rejection through analysis of stimulated porcine insulin secretion. Of these 17 mice, 4 received 20 million PBMCs and 13 received 40 million PBMCs. Although the data in Figure 5A displays an uptrend in stimulated porcine insulin secretion, it is important to understand that mice that displayed an absence of

porcine insulin at stimulated and basal levels were euthanized at the earlier time points, and only mice that did not reject were reanalyzed at later time points. Porcine insulin graft rejection was demonstrated by insulin secretion values at or near the lowest limit of detection on the ELISA curve for both basal (time 0) and stimulated (time 60) values.

In mice reconstituted with 20 million PBMCs (Figure 4A), there was no significant difference between basal and stimulated insulin secretion post-transplantation (pre-reconstitution) (p>0.05). Also, there was no significant difference between basal and stimulated porcine insulin secretion at all post-reconstitution time points (p>0.05). In mice reconstituted with 20 million PBMCs (Figure 4B), there was no statistically significant difference in the SI of any time points (p>0.05). It is important to mention that during analysis, 1 mouse reconstituted with 20 million PBMCs was removed from analysis during the 2-week post-reconstitution time point due to the presence of an extreme value. This is reflected in both the stimulated insulin secretion (Figure 4A) and stimulation index values (Figure 4B). Table 5 provides the data in pmol/L for insulin secretion at under basal (time 0) and stimulated (time 60) conditions for NPI transplanted mice reconstituted with 20 million PBMCs. Of the mice reconstituted with 20 million PBMCs, 25% displayed an absence of basal and stimulated porcine insulin secretion at 4-weeks post-reconstitution, and the two mice that were evaluated at 5-weeks post-reconstitution both demonstrated islet graft rejection (Table 5).

In mice reconstituted with 40 million PBMCs (Figure 5A), a significant difference was found at 4-weeks post-transplantation (**p<0.01) between basal and stimulated insulin secretion. However, statistically significant differences were not observed between the basal and stimulated insulin values during the post-reconstitution time points (p>0.05). In mice reconstituted with 40 million PBMCs (Figure 5B), there was a statistically significant difference in the SI between 4-

weeks post-transplantation and 6-weeks post-reconstitution (*p<0.05). No significant difference was found between the 4-weeks post-transplantation and 5-weeks post-reconstitution time points (p>0.0.5) (Figure 5B). Table 6 provides the data in pmol/L for insulin secretion at under basal (time 0) and stimulated (time 60) conditions for NPI transplanted mice reconstituted with 40 million PBMCs. Of the mice that received 40 million PBMCs, 30.8%, and 53.8% displayed islet graft rejection at 4- and 6-weeks post-reconstitution, respectively, indicated through the absence of porcine insulin secretion at basal and stimulated levels (Table 6).

When mice reconstituted with 20 and 40 million PBMCs were combined, a significant difference was found between the basal and stimulated porcine insulin secretion (*p<0.05) at 4-weeks post-transplant (Figure 6A). Similarly, no significance was found between the basal and stimulated insulin secretion post-reconstitution time points (p>0.05). When mice reconstituted with 20 and 40 million PBMCs were combined (Figure 6B), a significant difference was also found between the SI of 4-weeks post-transplantation and 6-weeks post-reconstitution (*p<0.05).

To assess the occurrence of GVHD in these mice post-reconstitution, non-fasting weight measurements were taken on the day of reconstitution (baseline) and subsequently, three times per week. The data indicates that mice did not decrease in weight by more than 20% from baseline values, indicating that GVHD may not have occurred within 6 to 7 weeks post-reconstitution (Figure 7A, B). Similar trends in weight were seen for mice reconstituted with 20 and 40 million PBMCs.

Morphological characterization of islet-bearing grafts revealed the presence of numerous human CD45^{+} immune cells (Figure 8A) at 10X magnification. Visual images confirmed the presence of dispersed porcine chromogranin A in the kidney capsule region of mice (Figure 8B) at 10X magnification. Upon closer examination using immunofluorescence, the co-localization

of human CD45$^+$ cells were observed infiltrating areas of chromogranin A positive staining at 20X (Figure 9A, C) and 40X (Figure 9B, D) magnification. No visual differences were observed in the co-localization of human CD45$^+$ cells in relation to Chromogranin A in mice reconstituted with 20 or 40 million PBMCs.
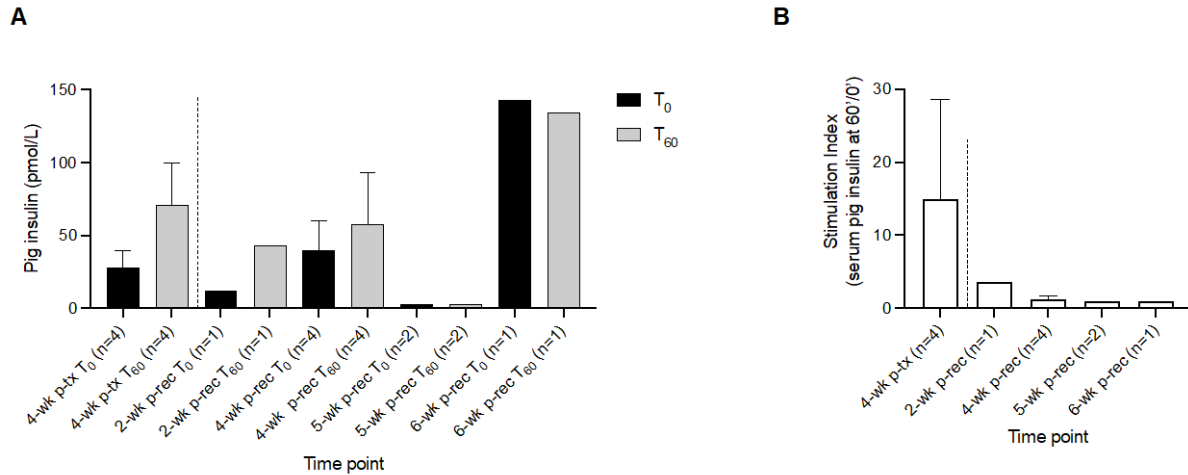
Figure 4: A) Porcine insulin stimulated secretion of NPI transplanted mice reconstituted with 20 million PBMCs. Black bars indicate basal porcine insulin secretion at time 0. Grey bars indicate stimulated porcine insulin secretion at time 60. No significant differences were found between anytime points. 1 mouse at 2-weeks post-reconstitution was removed from analysis due to the presence of an outlier. **B)** Stimulation index of NPI transplanted mice reconstituted with 20 million PBMCs. No significant differences were found between any time points. 1 mouse at 2-weeks post-reconstitution was removed from analysis due to an extreme value. (P-tx represents post-transplant (pre-reconstitution) whereas p-rec represents post-reconstitution). (A, B; ns $p > 0.05$).

Table 5: Data of porcine insulin stimulated secretion of NPI transplanted mice reconstituted with 20 million PBMCs obtained via ELISA. Values are represented as pmol/L and values were rounded to two decimal places. (* Indicates value at or near the lowest limit of detection, indicating rejection. NA Indicates IPGTT not performed. - Indicates that the animal was euthanized at the prior time point. Rem. indicates that the data set was removed due to outlier values. Min. represents minutes. P-tx represents post-transplant (pre-reconstitution) whereas p-rec represents post-reconstitution).

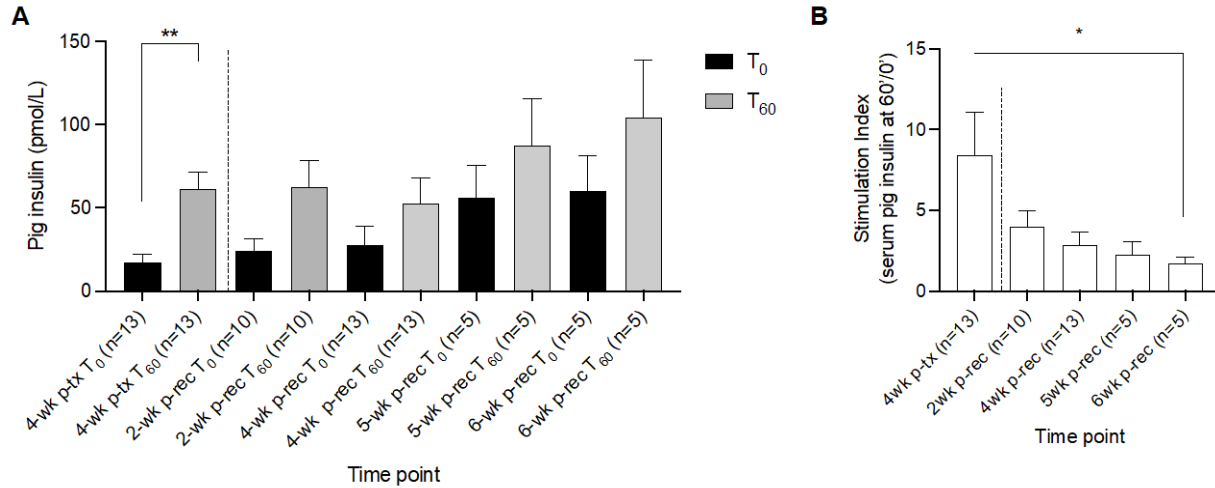| 4-wk p-tx		2-wk p-rec		4-wk p-rec		5-wk p-rec		6-wk p-rec	
0 min.	60 min.	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.
22.20	30.70	NA	NA	86.44	70.81	NA	NA	-	-
58.80	51.55	NA	NA	61.53	151.74	NA	NA	142.83	134.25
2.78	155.40	Rem.	Rem.	*	*	*	*	-	-
27.55	45.63	11.94	43.48	7.55	5.17	*	*	-	-

Figure 5: A) Porcine insulin stimulated secretion of NPI transplanted mice reconstituted with 40 million PBMCs. Black bars indicate basal porcine insulin secretion at time 0. Grey bars indicate stimulated porcine insulin secretion at time 60. A significant difference was found between the 4-week post-transplantation basal and stimulated insulin secretion values (**$p<0.01$). **B)** Stimulation index of NPI transplanted mice reconstituted with 40 million PBMCs. A significant difference was observed between the 4-week post-transplantation and 6-week post-reconstitution values (*$p<0.05$). (P-tx represents post-transplant (pre-reconstitution) whereas p-rec represents post-reconstitution). (A, B; ns $p>0.05$, *$p<0.05$, **$p<0.01$).

Table 6: Data of porcine insulin stimulated secretion of NPI transplanted mice reconstituted with 40 million PBMCs obtained via ELISA. Values are represented as pmol/L and values were rounded to two decimal places. (* Indicates value at or near the lowest limit of detection, indicating rejection. NA Indicates IPGTT not performed. - Indicates that the animal was euthanized at the prior time point. Min. represents minutes. P-tx represents post-transplant (pre-reconstitution) whereas p-rec represents post-reconstitution).

| 4-wk p-tx		2-wk p-rec		4-wk p-rec		5-wk p-rec		6-wk p-rec	
0 min.	60 min.	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.
14.05	34.45	NA	NA	*	*	NA	NA	*	*
25.67	62.47	NA	NA	*	*	NA	NA	*	*
6.25	42.72	NA	NA	16.31	36.55	NA	NA	*	*
2.78	38.15	7.56	8.44	2.78	14.10	2.78	25.28	*	*
2.78	37.19	8.60	62.60	18.27	89.02	37.91	71.60	44.37	155.33
2.78	95.3	2.78	30.24	2.78	32.18	*	*	-	-
2.78	51.15	2.78	11.97	*	*	*	*	-	-
10.22	43.70	*	*	*	*	*	*	-	-
2.78	8.26	33.07	62.36	25.90	46.15	70.92	73.77	57.27	55.45
35.55	81.87	31.20	134.04	82.78	134.96	120.91	208.58	168.49	190.45
25.23	120.66	33.68	149.65	52.20	142.29	126.44	184.40	123.22	309.38
36.69	46.67	40.93	73.44	15.05	28.61	23.39	70.42	25.85	111.08
57.55	135.61	76.04	88.66	133.33	146.38	169.56	232.01	162.80	212.13
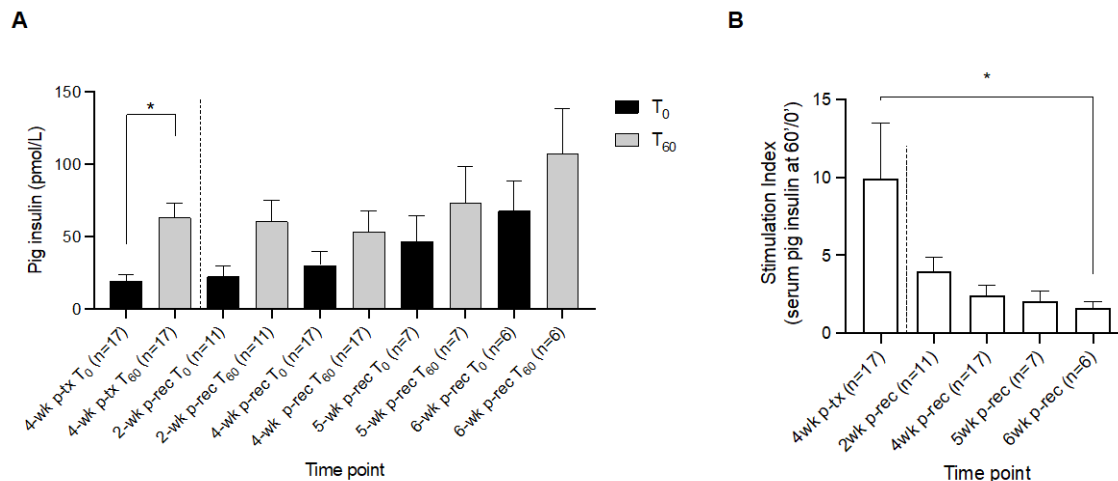
Figure 6: A) Porcine insulin stimulated secretion of NPI transplanted mice reconstituted with 20 and 40 million PBMC groups combined. Black bars indicate basal porcine insulin secretion at time 0. Grey bars indicate stimulated porcine insulin secretion at time 60. A significant difference was observed at the 4-week post-transplant insulin secretion values between basal (time 0) and stimulated (time 60) insulin secretion ($*p<0.05$). **B)** Stimulation index of NPI transplanted mice reconstituted with 20 and 40 million PBMC groups combined. A significant difference was found between the 4-week post-transplantation and 6-week post-reconstitution SI values (A, B; $*p<0.05$). (P-tx represents post-transplant (pre-reconstitution) whereas p-rec represents post-reconstitution). (A, B; ns $p>0.05$, $*p<0.05$).
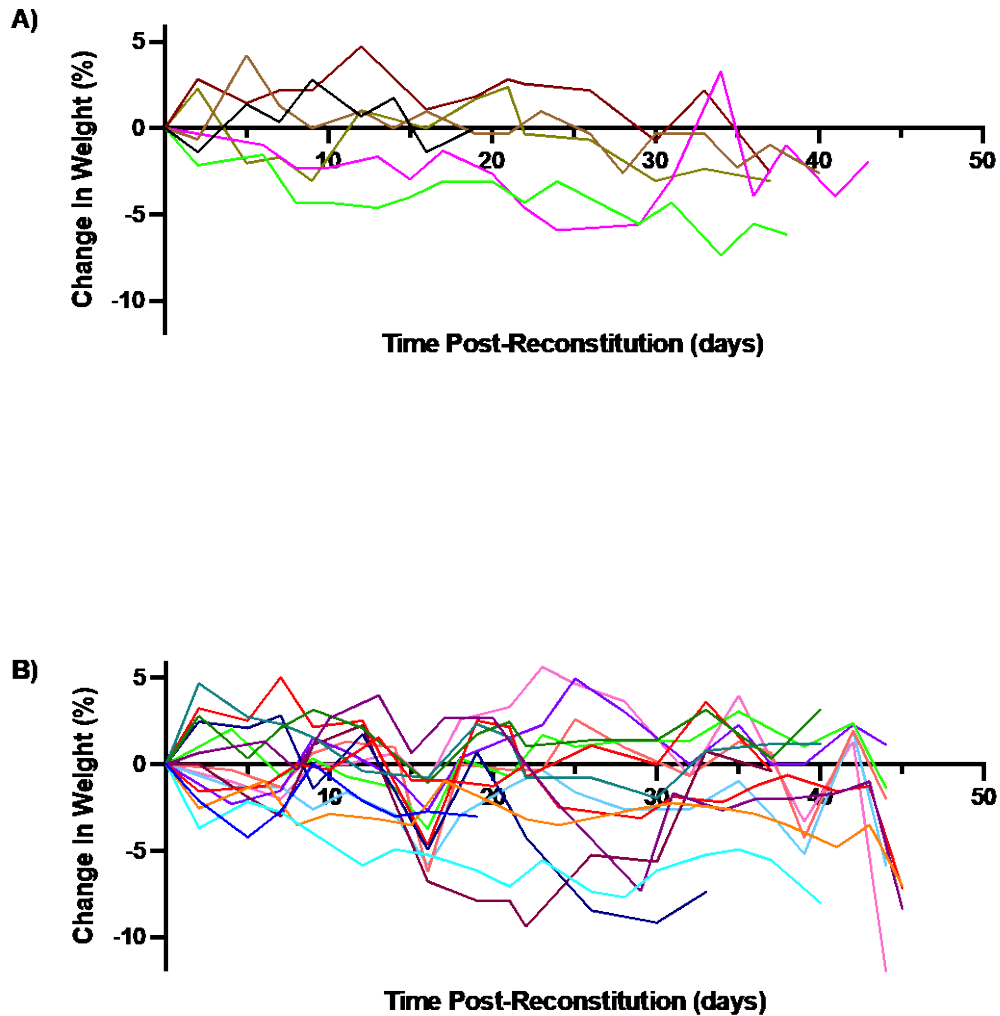
Figure 7: A) Percent change in weight of mice transplanted with neonatal porcine islets and reconstituted with 20 million PBMCs. A total of 6 mice were reconstituted with 20 million PBMCs. Mice retained healthy weight values over the duration of the study and no drastic decrease in weight was observed. **B)** Percent change in weight of mice transplanted with neonatal porcine islets and reconstituted with 40 million PBMCs. A total of 15 mice were reconstituted with 40 million PBMCs. Although there is a downward trend in percent change in weight, mice did not lose more than 20% of their weights compared to baseline, concluding that this external symptom of GVHD was absent.
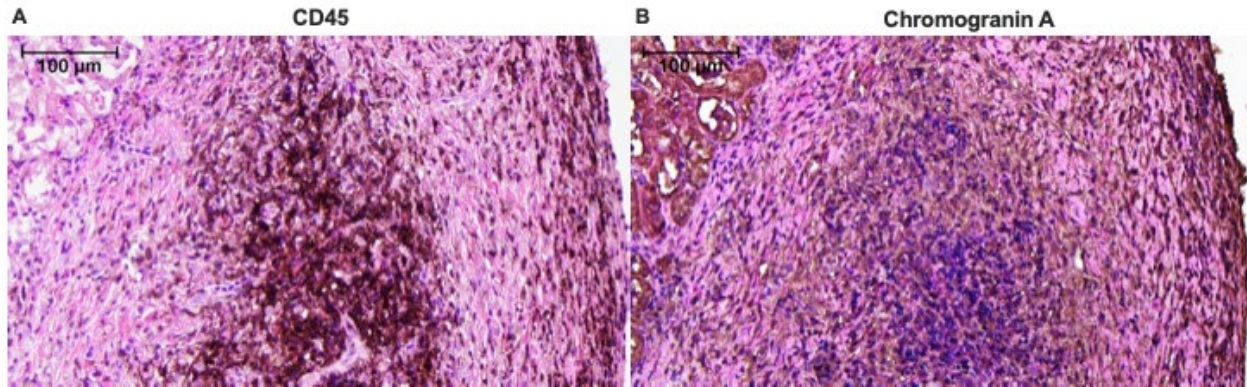
Figure 8: ABC-DAB staining of the islet bearing-kidney graft in NPI transplanted mice reconstituted with 20 million PBMCs in an animal in cohort 3 euthanized at 37 days post-reconstitution. Representative images are displayed showing CD45$^+$ cells (**A**) and Chromogranin A (**B**) in consecutive sections. Images are displayed at 10X magnification and positive staining is indicating in brown.
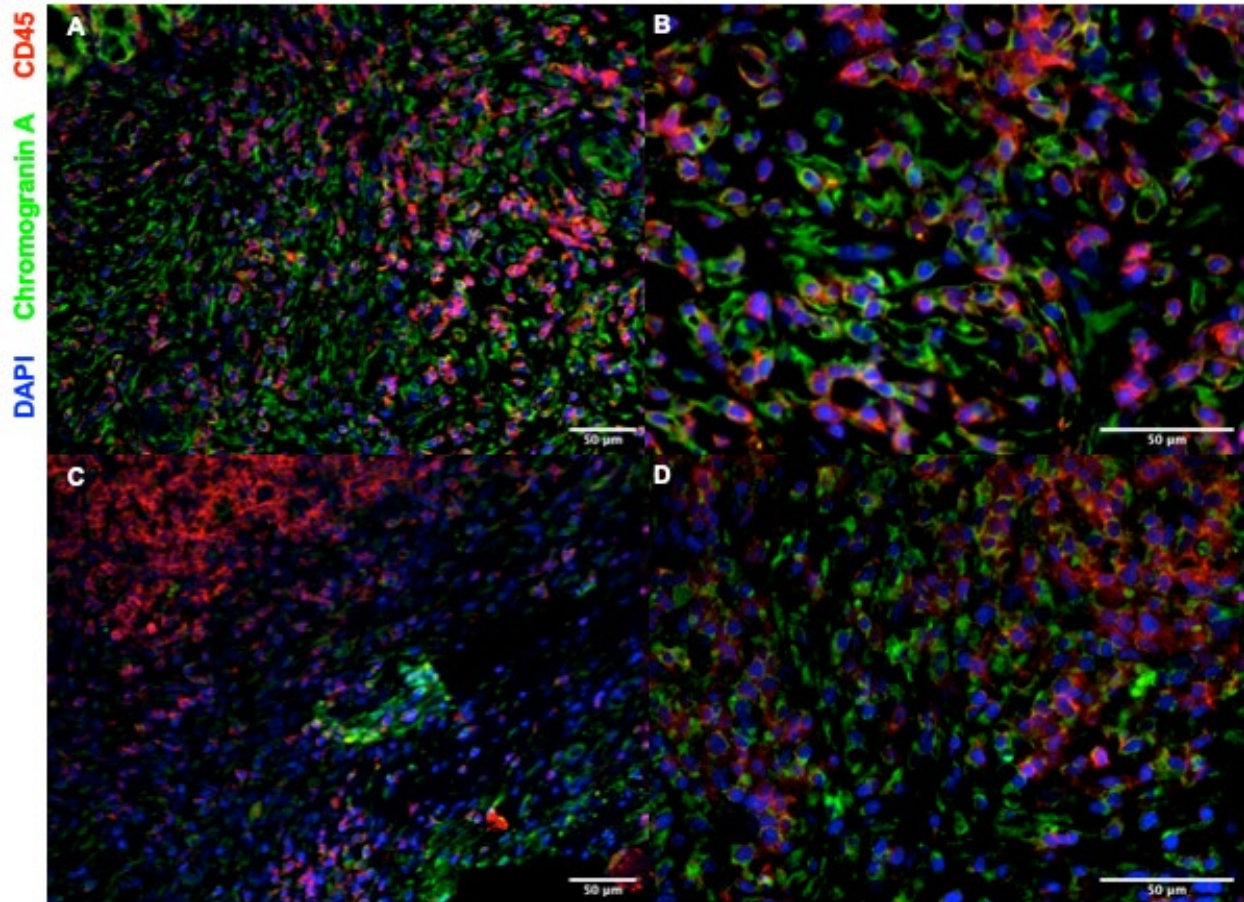
Figure 9: Immunofluorescence staining of NPI transplanted mice reconstituted with 20 million PBMCs in an animal in cohort 3 euthanized at 37 days post-reconstitution (**A & B**), or 40 million PBMCs in an animal in cohort 4 euthanized at 37 days post-reconstitution (**C & D**). Image displays the observed co-localization of human CD45^{+} immune cells (red) infiltrating the porcine islet graft region stained with chromogranin A (green). The nuclei of individual cells are stained with DAPI (blue). No visual differences were observed in the number of human immune cells infiltrating the graft region, and dispersed chromogranin A staining is present in both mice. Images display the graft region at 20X (A, C) and 40X (B, D) magnification.

2-3.4 Analysis of Stem Cell-Derived Islet Transplantation and Reconstitution:

A total of 5 mice from two cohorts were transplanted and 4 mice survived the transplantation procedure. Out of these mice, 3 became euglycemic and were used as recipients of human PBMC reconstitution. All SC-β transplanted mice used for reconstitution achieved euglycemia within 16 weeks of transplantation, although mice in cohort 2 achieved euglycemia earlier. Ultimately, 2 reconstituted mice displayed metabolic rejection as indicated by hyperglycemia. The mouse that did not revert to the hyperglycemic state was excluded in this study. In these experiments, both cohorts received human PBMCs from donor 2.

Prior to reconstitution, metabolic follow-up of non-fasting blood glucose was measured weekly, along with alternative week readings of fasting blood glucose (Figure 10A) after removal of the LinBit. Mice transplanted with SC-βs were reconstituted after 16- and 12-weeks post-transplantation with 40 million PBMCs. PBMCs were obtained either fresh or thawed from donor 2. The kinetics of SC-β rejection in mice was determined to provide an initial understanding of the immunogenicity of stem cell sources of islets. Results indicate that these mice displayed islet graft rejection due to PBMC reconstitution at 21- and 23-days post-reconstitution (Figure 10B), which could indicate the possibility of lowered immunogenicity of SC-βs.

IPGTTs were performed at 8- and 12-weeks post-transplant to test the *in vivo* functionality of SC-βs. Results indicate that mice transplanted with SC-β display insulin secretion at basal and stimulated levels (Figure 10C). In addition, the stimulation index also points to the fact that these mice displayed increased insulin secretion under stimulated conditions (Figure 10D). Though, statistical analysis could not be performed in this group due to the small sample size.

Immunofluorescent staining indicated the presence and co-localization of human CD45$^+$ cells and human insulin at 10X and 20X magnification (Figure 11A, B). DAB staining of consecutive tissue sections displayed positive staining for human insulin and human CD45$^+$ cells and showed the morphological characteristics of the murine kidney and islet graft (not displayed).
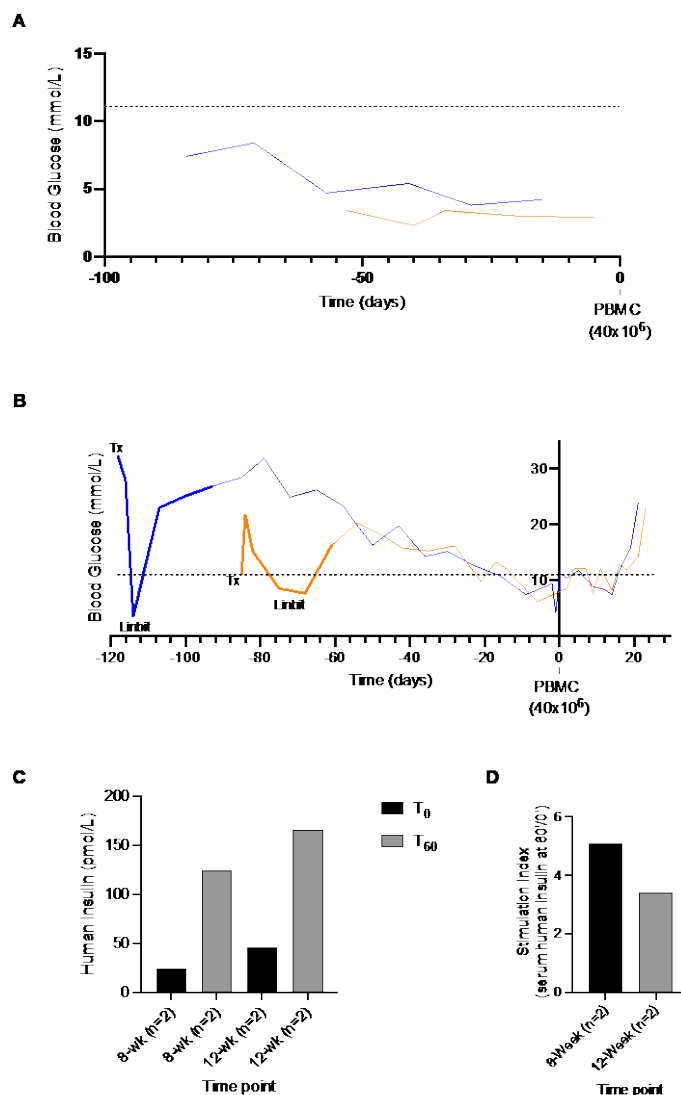
Figure 10: A) Fasting blood glucose of mice transplanted with SC-β prior to reconstitution. The functionality of SC-β transplanted mice were assessed under fasting conditions as an indirect measure of graft function. Blue line represents cohort 1 and orange line represents cohort 2. **B)** Non-fasting blood glucose of mice transplanted with SC-β and reconstituted with 40 million PBMCs. 2 mice were assessed for metabolic graft rejection as indicated by a reversal to hyperglycemia. Bold lines indicate the period in which mice contained LinBits. Both mice received an intraperitoneal injection of 40 million human PBMCs at day 0 and displayed graft rejection at 21 and 23 days. Blue line represents cohort 1 and orange line represents cohort 2. **C)** Intraperitoneal glucose tolerance test (IPGTT) assessing basal (time 0) and stimulated (time 60) human insulin secret at 8- and 12-weeks post-transplantation. A total of 2 mice were assessed. Black bars indicate basal human insulin secretion at time 0. Grey bars indicate stimulated human insulin secretion at time 60. **D)** Stimulation index (SI) of human insulin secretion represented as stimulated (time 60) over basal (time 0) insulin release. A total of 2 mice were used for analysis. Black bar indicates 8-week and grey bar indicates 12-week SI. (C, D; ns p>0.05).
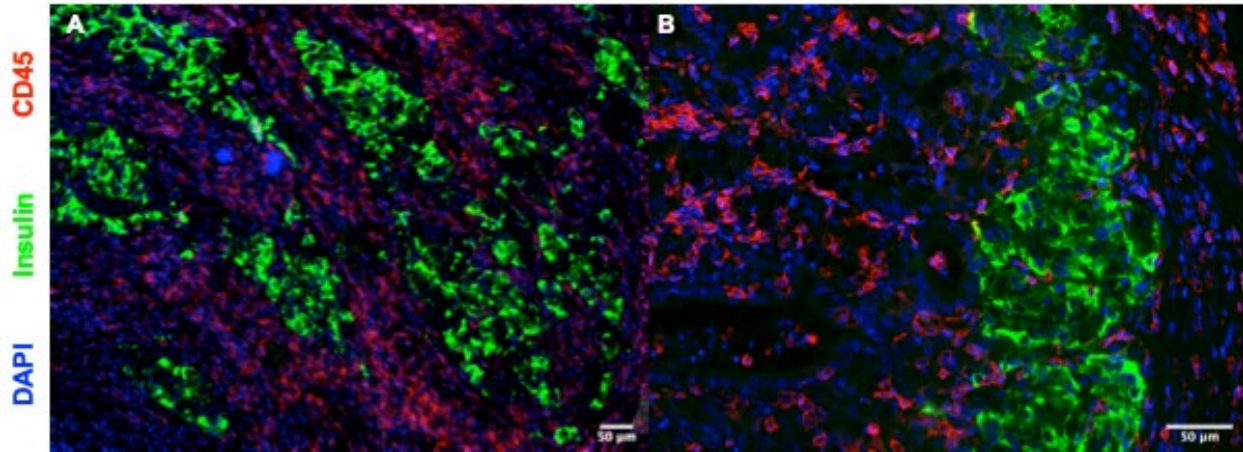
Figure 11: Immunofluorescence staining of SC-β transplanted mice reconstituted with 40 million PBMCs (**A & B**). Image displays the observed co-localization of human CD45⁺ cells (red) infiltrating the human islet graft region stained with insulin (green). The nuclei of individual cells are stained with DAPI (blue). Images display the graft region at 10X (A) and 20X (B) magnification of the islet-kidney graft in cohort 1 euthanized at 21 days post-reconstitution.

2-3.5 Comparison of Human Islet and Stem Cell-Derived Islet Reconstitution:

To determine if there is a difference in the kinetics of human PBMC driven rejection in HI and SC-β transplanted mice, the median time points of rejection from both groups were compared using a Kaplan-Meier Log-rank (Mantel-Cox) test. Mice from both groups that were given 40 million PBMCs were compared, and results showed that there was a statistically significant difference (*$p<0.05$) between the median rejection rate of both groups (Figure 12A).

As determined previously, there was no statistically significant difference between the median rejection rates of HI transplanted mice reconstituted with 40 and 60 million PBMCs ($p>0.05$) (Figure 2C). Therefore, the median timepoint of rejection of these mice were also compared to SC-β transplanted mice reconstituted with 40 million PBMCs. Similarly, a statistically significant difference (*$p<0.05$) was found when comparing mice from these groups (Figure 12B).

A
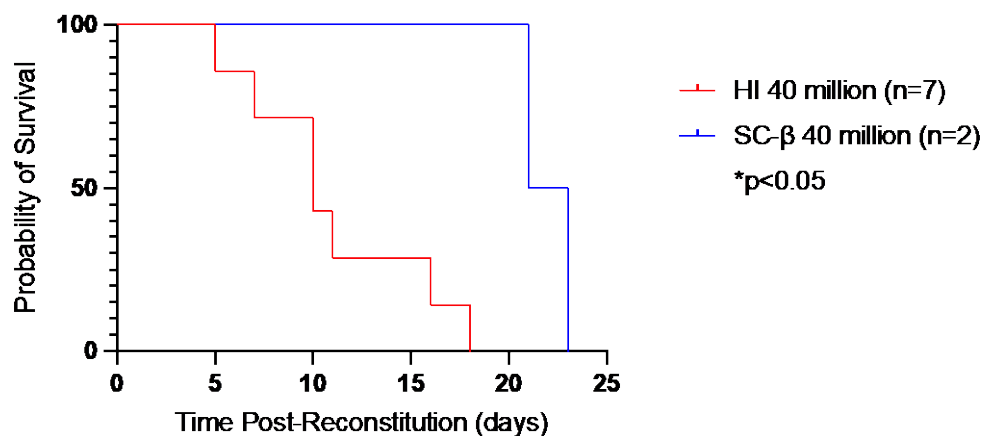
B
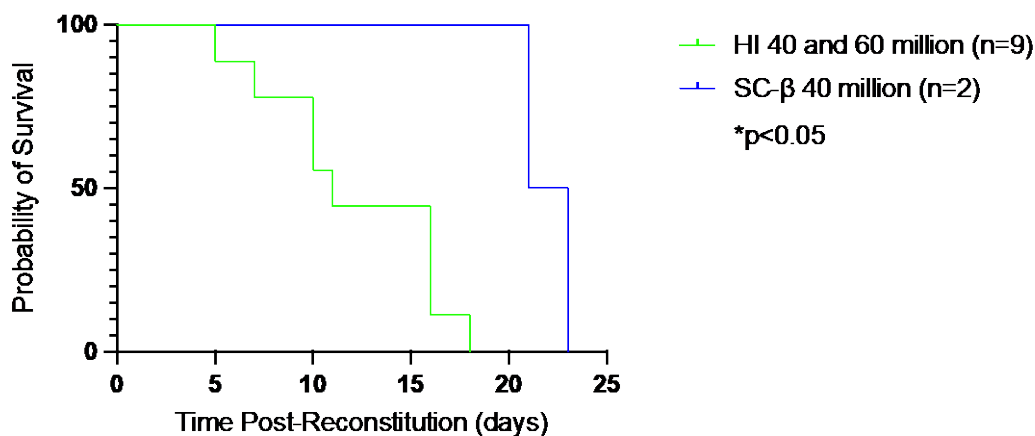
Figure 12: A) Kaplan-Meier Log-rank (Mantel-Cox) test comparing the median rejection rates of human islet (red) and stem cell-derived islet (blue) transplanted mice reconstituted with 40 million PBMCs. A significant difference was found (*$p < 0.05$) between these groups. **B)** Kaplan-Meier Log-rank (Mantel-Cox) test to compare median rejection rates of human islet (green) transplanted mice reconstituted with either 40 or 60 million PBMCs combined, and stem cell-derived islet (blue) transplanted mice reconstituted with 40 million PBMCs. A significant difference was found between these groups. (A, B; *$p < 0.05$).

2-4 DISCUSSION:

Our understanding of the mechanisms of islet graft rejection in humans is largely due to studies using rodent models. However, directly converting experimental data from mice to human pathophysiology is unwarranted due to several interspecies differences. It is important to consider the differences between human and murine biology, mainly immune system function [232]. A key difference in the immune system between the two species is the presence of reactive memory T-cells in humans that may be absent in mice [185]. In addition, the lack of functional Toll-like Receptor 10 family (TLR10) is absent in mice, but present in humans, highlighting the differences in the innate immune system between the species. [179, 186] This emphasizes the need to develop a mouse model that can recapitulate the human immune system and survive for an extended period to conduct long-term immunological studies. Reconstitution of NSG-MHC I/II DKO mice allow for that goal as the limitations previously posed from GVHD are no longer a major concern [179]. Our primary rationale for the development of this humanized mouse model is to allow for long-term studies that would allow researchers to develop new or improve existing strategies to prevent islet graft rejection.

In a previously developed humanized murine model, $H2^d$-$Rag2^{null}$ $IL2rg^{null}$ mice have successfully become engraftment with human PBMCs. However, the authors report that the model requires pre-conditioning with sub-lethal levels irradiation to exhaust mouse macrophages [233, 234]. Although this model may be used for islet graft rejection studies, the time commitment and machinery required to create a human-mouse chimeric model is meticulous. In addition, this method does not guarantee the complete removal of the host immune system which can potentially limit the study of human immune system mediated islet graft rejection. In another report, Banuelos et al. [235] successfully demonstrated the human PBMC mediated rejection of

human and HLA-transgenic mouse allografts transplanted in the spleen in NOD-*Rag1^{null}* *Prfr^{null}* mice. Later, King et al. [178] demonstrated the use of a new model for the study of human immune system function. This model consisted of using NOD.cg-prkdc^{scid} *ILrg^{tm1Wjl}*/SzJ mice transplanted with 3000-4000 IEQ of human islets and reconstituted with human PBMCs from normal donors [178]. Although these preliminary studies provided a gateway to understand human immune system function in a working model, the researchers were not able to conduct extended graft rejection studies due to the possibility of the development of GVHD in these models. Since these mice still contain MHC class I and class II, it was still possible for the administered human PBMCs to recognize and attack the foreign mouse tissue limiting the timeframe for conducting graft-rejection studies.

Due to the limitations posed by GVHD from previous murine models, a new strain of mice was necessary for the investigation of long-term graft rejection studies. Brehm et al. [184] were one of the first groups to investigate the potential of a new strain of mice that could become engrafted with high levels of PBMCs without being compromised due to GVHD [184]. Their experiments used NSG-*(K^bD^b)^{null}(IA^{null})* as well as the NSG-*(B2M)^{null} (IA IE)^{null}*, both of which are stated to lack MHC class I and II. Similarly, our experiment used the same strain as the former mentioned mouse.

The goal of this thesis is to develop a humanized mouse model that could complement the study of islet graft rejection through an extended timeframe, in the presence of a human immune system. Our experiments were unique as they demonstrated the efficacy of transplanting different β-cell sources in diabetic or non-diabetic mice and using different approaches to investigate graft rejection. Our initial experiment was geared towards investigating the native immune system in these mice. Using immunofluorescent staining, we demonstrated that these

mice do not possess a leaky immune system as the presence of murine CD4$^+$ and CD8$^+$ T-cells were found to be absent. Although staining for CD68 macrophages was positive, they are stated to be defective in function due to the alleles found in the NOD genetic background [184, 241]. These initial experiments confirmed the immunodeficiency in this strain and allowed us to conduct transplantation and subsequent reconstitution experiments.

Our experiments have demonstrated the feasibility and functionality of transplanting HIs, NPIs, and SC-βs in these mice. Our experiments with HI and SC-β have shown the ability of these cell sources to reverse diabetes in this model. Further, our experiments have demonstrated the functionality of NPIs in non-diabetic, naïve mice from their capacity to secrete insulin under basal and stimulated conditions after 4-weeks. These observations provided baseline values to compare to post-reconstitution and further evaluate the efficacy of reconstitution.

Our results from HI transplants have shown that mice reconstituted with 40 million PBMCs display islet graft rejection as early as 5 days and as late as 18 days. This was in line with the observations of Brehm et al. [184]. Their study transplanted 4000 IEQ of human islets in the intrasplenic region and mice received IP injections of HLA-mismatched human PBMCs. The authors observed that ¾ mice that received 50 million human PBMCs reverted to hyperglycemia within 3-4 weeks, indicating islet graft rejection [184]. Another study by King et al. [178] used NOD mice bearing the *IL2rg^null* gene as recipients for intrasplenic transplantation of 3000-4000 IEQ of human islets. Mice received IV PBMC delivery either on the same day of transplantation, or at 37 days post-transplantation [178]. In both groups, the metabolic rejection profile was evaluated. The authors found that simultaneous IV injections of 20 million PBMCs led to intrasplenic human islet graft rejection within 21 days. Similarly, they reported that 2 out of 3 mice that were reconstituted at 37 days post-transplant displayed hyperglycemia at 21 days post-

reconstitution [178]. Although our observations are in line with both studies, it is important to acknowledge the difference in the location of transplanted cells as well as the dosage of administered PBMCs. Furthermore, King et al. [178] have demonstrated that IV injections of PBMCs provided an optimal route of engraftment. Although we have not examined the effects of alternative routes of administration of PBMCs, it may be worthwhile to test this in the future for further optimization of engraftment. In our other cohort of HI transplanted mice reconstituted with 60 million, we have observed islet graft rejection, indicated by a reversal to hyperglycemia at 16 days in both mice. Though our studies provided an initial observation of the timeframe of rejection in these mice, using a larger sample of mice for both groups would provide more information about the timeline of rejection of human islets using different doses of PBMCs. In addition, although we found no statistically significant difference in the survival curve of HI transplanted mice reconstituted with 40 and 60 million PBMCs, using a larger sample size in both groups would provide more conclusive results.

Our studies using NPIs have demonstrated the usefulness of this model in non-diabetic, naïve mice. As NPIs contain very few insulin producing cells, the cellular aggregates of β-cells would require an extended period to normalize diabetic mice [238, 239]. Therefore, an alternative method to evaluate rejection in naïve mice was necessary. Assessing the insulin secretion of naïve mice at specific time points prior to and post-reconstitution provided an analytical and quantitative method to evaluate the kinetic rejection profile in mice. Our studies evaluated the ability of mice to secrete porcine insulin under fasted (time 0) and stimulated (time 60) conditions. This demonstrates the *in vivo* functionality of transplanted NPIs in mice over successive periods and allowed us to evaluate the decrease in functionality over time. Calculation of the SI provided an additional measure to evaluate the degree of insulin secretion. Comparing

the SI prior to and post-reconstitution provides an objective measure to evaluate metabolic decline in NPI function. It may be advantageous for future studies to evaluate the rejection profile of NPIs in naïve mice using a more extended timeline as allowing NPIs to mature *in vivo* may provide future researchers with more conclusive observations. Nonetheless, our experiments provide a benchmark to further evaluate the rejection profile of NPIs.

As previously mentioned, 30.8%, and 53.8% of mice transplanted with NPIs and reconstituted with 40 million PBMCs displayed islet graft rejection at 4- and 6-weeks post-reconstitution, respectively. This is demonstrated through a functional decline of stimulated porcine insulin release across successive weeks through basal and stimulated porcine insulin secretion at or near the lowest limit of detection on the ELISA curve. Since 53.8% of mice displayed complete NPI rejection indicated through an absence in basal and stimulated porcine insulin secretion at 6-weeks post-reconstitution, it may be beneficial to experiment on longer timeframes post-reconstitution to further display a decline in stimulated insulin secretion in a larger percentage of mice. It is also important to understand that mice that displayed graft rejection through an absence of porcine insulin secretion under basal and stimulated conditions were euthanized and not assessed at later time points. Therefore, there appears to be an uptrend in insulin secretion due to the lower number of mice analyzed at later time points.

In NPI transplanted mice reconstituted with 40 million PBMCs, we have observed a decreased in the SI over successive time points post-reconstitution, which indicates metabolic decline of NPI function *in vivo*. The data has indicated a significant difference between the 4-week post-transplantation and 6-week post-reconstitution timepoint. This observation demonstrates that the 6-week post-reconstitution timepoint may be a key timeframe in which we begin to observe NPI graft rejection in a majority of transplanted mice provided with 40 million

PBMCs. Though 25% of mice reconstituted with 20 million PBMCs displayed islet graft rejection at 4-weeks post-reconstitution, it is important to recognize that a smaller sample size was analyzed with 20 million PBMCs (n=4) compared to those given 40 million PBMCs (n=13). Therefore, these observations may be strictly reliant on the dose of administered PBMCs as well as the number of transplanted cells. It is important to use a larger sample of mice for future *in vivo* NPI rejection studies to not only reinforce our observations, but also to examine wider parameters in PBMC doses and number of cells transplanted.

Our study also briefly demonstrated the efficacy of using this mouse model to examine the rejection profile of stem cell sources of islets. However, this data is preliminary as a small sample of mice were analysed for this purpose. Prior to reconstituting SC-β transplanted mice, an extensive analysis of the cell composition of SC-βs and outcome of transplantation results was performed, which can be found in Appendix A. In our preliminary reconstitution results, we examined that SC-β transplanted mice demonstrated rejection as indicated by a reversal to hyperglycemia when reconstituted with 40 million PBMCs, within 23 days post-reconstitution. Further, the comparison of the median timepoint of rejection between HI and SC-β transplanted and reconstituted mice demonstrated a significant difference between the groups. However, it must be noted that due to the small sample size of mice used in each experiment, the results from this comparison could be strengthened or yield different results when using a larger sample size in each experimental condition. Nonetheless, this preliminary data seems to be in line with observations regarding the reduced immunogenicity of stem cell derived tissues, although not conclusive as the maturation of stem cells *in vivo* may reduce their lowered immunogenicity [212, 236, 237]. Further studies with SC-β transplantation as well as more careful and direct evaluations are required to characterize the immunogenicity of SC-βs. As well, using a larger

sample size to investigate the rejection profile of SC-β transplanted mice would provide more consolidative results.

Although the usage of NSG-MHC I/II DKO mice have provided us with significant insight on the rejection profiles of HIs, NPIs, and SC-βs, it is essential to consider the limitations of this study. It is important to acknowledge that human PBMCs do not provide researchers with a complete understanding of human immune function. Human PBMCs are limited since they do not contain neutrophils, eosinophils, and platelets, which are essential components of human blood [229, 230]. Furthermore, human blood is neutrophil rich, with 50-70% neutrophils and 30-50% lymphocytes [232]. Although the role of neutrophils in graft rejection has not clearly been established, our understanding of human immune mediated graft rejection may not be complete. Another limitation of this model was that the interspecies sex differences between mice were not studied. Although our model only used male mice, it is important to test these parameters using female mice as they may respond differently in terms of the timeline of rejection. This would provide researchers with valuable insight in terms of the heterogeneous outcomes that may be expected during clinical translation. Furthermore, the fact that only female PBMC donors were used may also limit our observations. PBMCs of female donors have been demonstrated to contain significantly greater mitochondrial function compared to males, along with higher ATP levels in female PBMCs [240]. Future experiments should take advantage of using both human male and female PBMC donors from a variety of backgrounds to better understand the immunological kinetic profile of islet graft rejection as well as the sex-associated differences in PBMCs. Another limitation of this study was that the timepoint of engraftment was not evaluated in the reconstituted mice. Better understanding of when human PBMCs effectively engraft into the murine model will allow the establishment of more controlled and effective evaluation of

data. To have a clearer understanding of human immune system function, it is imperative to further evaluate the degree and time points of engraftment of the human immune system in this model. Lastly, it is important to mention the limitations posed using IPGTTs to evaluate stimulated insulin secretion. One limitation of using IPGTTs in this study is that we are limited in identifying the response of other circulatory hormones. For example, incretin hormones, such as GLP-1 (glucagon-like polypeptide 1) and GIP (glucose-dependent insulinotropic polypeptide), exert their effect during oral food consumption. These hormones play vital roles in controlling the secretion of insulin, glucagon, and somatostatin [268]. The incretin effect on glucose clearance is not observed in subjects undergoing IPGTTs, whereas it can be observed during the OGTT response. Further, it is suggested that there may be a difference in insulin secretion during the OGTT and IPGTT, which may be due to incretin hormones [269]. Therefore, future studies should aim to understand the role of the incretin effect in glucose clearance and corresponding differences in stimulated insulin secretion in mice during instances of islet graft rejection. Such data may provide invaluable information to better understand potential changes in the hormonal response during instances of islet graft rejection.

The potential of this model for future application poses unlimited possibilities. Using this model to test healthy versus diabetic PBMC donors would provide researchers with supplementary insight on the pathology of type 1 diabetes. The usage of low immunogenicity cell sources such as SLA knockout porcine islets or hypoimmunogenic stem cells could provide an important understanding of immunogenicity of these different cells. Further, this model could be applied to well-established methods to further study the delay in graft rejection. For instance, using encapsulation or localized immunosuppressive drug delivery strategies could provide immense insight on how well these methods delay islet graft rejection. In turn, these studies

could strengthen our understanding of islet graft rejection using a reliable and recognized model, which could allow researchers to fine-tune their protocols to further delay islet graft rejection. This model will also provide researchers with an understanding of how patients may respond in clinical studies. The large heterogeneity observed in mice of this model provides valuable insight on what may be the case in humans. There is immense applicability of this model and future research will only broaden our understanding and allow for improved islet transplantation results in the clinic.

CHAPTER THREE

GENERAL DISCUSSION AND CONCLUSION

3-1 GENERAL DISCUSSION:

Type 1 diabetes is a chronic condition in which the pancreatic β-cells are targeted for autoimmune destruction, resulting in a decrease or lack of insulin secretion [242]. Ultimately, if left untreated, the resulting hyperglycemia and insulin resistance contributes to a wide range of micro and macrovascular complications such as retinopathy, neuropathy, nephropathy, stroke, coronary artery disease and peripheral artery disease [41]. The discovery of insulin in 1921 has revolutionized the treatment for type 1 diabetes. However, the problem remains that insulin is only a treatment option and not a cure for diabetes [2]. In the year 2000, a hallmark study in Edmonton allowed 7 patients to attain insulin independence for a period of 1 year through a novel islet transplantation and immunosuppressive regimen approach (and 100% achieved sustained insulin independence after 1 year) [82]. However, the diminishing 5-year insulin independence rate of 10% made it clear that further refinement in the approach was necessary [103, 105].

Islet transplantation is a promising alternative to conventional insulin injections however, limitations remain. The patient selection process is rigorous, and the treatment option serves as a last resort after all previous treatment options have been exhausted [243]. The usage of cadaveric human islets is not sustainable nor the optimal cell source for treating patients with type 1 diabetes. Cadaveric human islets may be exposed to multiple comorbidities that may limit them to being the most optimal treatment source [244]. Further, controlling the immune response of transplant patients is necessary to prevent transplant rejection. However, the use of systemic immunosuppressive agents may create multiple complications that arise throughout the course of treatment [245]. For islet transplantation to be a more widespread treatment option for patients with type 1 diabetes, these limitations must be addressed.

The usage of alternative β-cell sources including stem cell-derived islets and neonatal porcine islets pose promising replacements to conventional cadaveric human islet usage. Porcine sources of islets can be easily obtained, and the environment can be controlled for optimal, pathogen-free breeding [246]. Stem cell-derived islet sources could be obtained from living human donors and cultured to produce a constant supply of islets [247]. Further, the ease of genetic manipulation in both sources may allow for improved insulin secretion capability and reduced immunogenicity [246, 247]. However, before widespread clinical adoption of alternative β-cell sources is to occur, it is important that islet graft rejection of human islets and alternative β-cell sources are studied in a reliable model that realistically mimics the human immune system.

The primary goal of this thesis is to establish a reliable humanized mouse model that can reproduce a functional human immune system. To further understand the process of islet graft rejection and to develop new methods to prolong or delay islet graft rejection, it is first essential to have a working model that reliably reproduces a functional decrease in insulin secretion and a reversal to the hyperglycemic state, displaying metabolic decline of graft functionality. As well, it is important to visually characterize human immune cell infiltration of the islet graft region to confirm the co-localization and presence of human immune cells in relation to insulin producing cells. This has been made possible through advancements in our understanding of the human immune system, different strains and applications of mice, and the variety of already established quantitative and qualitative analytical methods.

In Appendix A, preliminary research with stem cell-derived islets broadened our understanding of the necessary cell batches required to maintain euglycemia in two different strains of mice. The research also evaluated the differences in insulin secreting capability and *in vivo* maturation of stem cell-derived islets that co-express markers of mature β-cells. This

understanding will allow researchers to better optimize the conditions required for stem cell-derived islets to differentiate into mature insulin producing cells with paramount performance. With this knowledge, researchers will be able to produce more optimal low immunogenicity insulin producing stem cells differentiated from stem cells that may translate into improved results in the clinic.

In the past, it has been discovered that mice bearing the *IL2rg^null* gene allowed for superior engraftment and a closer mirror of the human immune system than all previous models of humanized mice [179, 184, 248 – 250]. In NOD mice that contained this null gene and was engrafted with human peripheral blood leukocytes displayed engraftment of functional human CD3$^+$ T-cells within one week. However, these mice remained limited in their potential as they would develop graft versus host disease within 4-8 weeks of reconstitution [179]. Therefore, researchers were limited in the timeframe in which they could conduct studies in relation to the human immune system. This limitation was overcome through the development of NSG mice that lacked major histocompatibility complex (MHC) class I and II. The use of this new model now allowed researchers to conduct immunological studies over an extended period [179, 223].

In our experiments, we used the new NSG strain that contained the MHC class I and II double knockout to conduct long-term studies of islet graft rejection. Our objective was to observe the differences in islet graft rejection using human, stem cell, and neonatal porcine islets and characterize an objective method to identify graft rejection using both diabetic and naïve mice. Previous studies by Brehm et al. [184] have also used this same mouse model to study the process of human islet graft rejection. Although Brehm et al. [184] studied human islet graft rejection using two different strains of NSG mice, both containing the MHC class I/II double knockout, our experiments were limited to using a single strain. Further, Brehm et al. [184] were

able to identify the degree of engraftment of human immune cells in these mice at different time points. This was not done in our experiments but replicating the observations as seen in previous experiments will provide researchers with a better understanding of the degree of engraftment in these mice. Further, it is important to mention the differences in the location of the transplanted human islets, as well as the dose of human peripheral blood mononuclear cells (PBMC) administered to the mice. The study by Brehm et al. [184] conducted intrasplenic transplantation of 4000 islet equivalents (IEQ) of human islets and administered 50 million PBMCs to mice. On the other hand, our experiments used 2000 IEQs of human islets transplanted under the kidney capsule and administered either 40 or 60 million PBMCs to these mice. Nonetheless, our studies observed similar findings to those of Brehm et al. [184]. Our results display that 100% of mice reconstituted with 40 or 60 million PBMCs displayed islet graft rejection, as indicated by a reversal to the hyperglycemic state, by 18 days post-reconstitution (around 3-weeks). Similarly, the study by Brehm et al. [184] found that ¾ mice displayed islet graft rejection at 3-4 weeks. The differences may be due to the number of human islets transplanted, the location of the transplant, and the purity and preparations of islets.

In addition to human islet graft rejection studies, Brehm et al. [184] showed that the majority of two strains of mice that were used did not develop GVHD like symptoms (i.e., weight loss more than 20% of baseline, fur-loss, hunched posture, reduced mobility, and tachypnea) for up to 125 days post-reconstitution. Specifically, Brehm et al. [184] showed that 13/15 of the same mouse strain that was used in our studies did not develop GVHD like symptoms at 125 days post-reconstitution. In our studies, we did not observe any GVHD-like symptoms in mice 46 days post-reconstitution. Although our studies did not conduct an observation of GVHD like symptoms for an extended timeframe, the fact that mice did not

develop GVHD like symptoms at 6-weeks post-reconstitution poses promising results. In the future, it would be wise to conduct observations over longer timeframes to confirm the absence of GVHD in these mice.

In another study, NOD mice bearing the *IL2rg^{null}* gene were used as recipients of human islet transplantation. Although these initial studies used mice that did not contain the MHC class I/II double knockout, the experiments still hold value in expanding our understanding of islet graft rejection in a murine model. King et al. [178] transplanted 3000-4000 IEQ of human islets in the intrasplenic region and administered 20 million PBMC via intravenous (IV) injection. In this study, the authors either administered PBMCs at the same time as transplantation, or at 37 days post-transplantation. They found that when PBMCs were administered at the same time as transplantation, 8/8 mice reverted to hyperglycemia, indicating human T-cell mediated graft rejection at 21 days post-reconstitution. In mice that were reconstituted 37 days post-transplantation, the authors observed that 2/3 mice rejected the islet graft [178]. Similarly, our experiments observed that 7/7 mice that were transplanted with 2000 IEQ of human islets and reconstituted with 40 million PBMCs displayed islet graft rejection at or before 18 days post-reconstitution. When these mice were provided with 60 million PBMCs, 2/2 of the mice displayed graft rejection at 16 days post-transplantation. King et al. [178] also examined different routes of PBMC administration including intravenous, intraperitoneal, and intrasplenic administration. They found that intravenous injection of PBMCs resulted in significantly higher average percentages of $CD45^+$ cells in blood compared to the other two routes [178]. Although our study used the intraperitoneal injection route, it may be beneficial to compare the rate of engraftment between intravenous and intraperitoneal routes in the future to obtain more optimal results. Further, it may also be worthwhile to investigate the difference between simultaneous

transplantation and reconstitution in comparison to transplantation and subsequent reconstitution at a different timepoint.

Porcine islets provide another potential avenue as an alternative to human sources of islets. Porcine islets have similar morphology and physiology to human islets, and specifically, neonatal porcine islets (NPI) are an ideal future cell source for transplantation in humans due to their hypoxia-resistant characteristics, easy isolation process, and their ability to remain robust in culture conditions [78]. However, a critical issue that prevents the widespread adoption of porcine islets is graft rejection.

Previous work has used immunodeficient humanized NSG diabetic mice transplanted with neonatal porcine islets. The mice displayed euglycemia following transplantation and were reconstituted with PBMCs either from healthy donors or donors with type 1 diabetes. Mice were euthanized at 1-, 2-, and 3-weeks post-reconstitution and histology for insulin positive cells was observed in these mice. At 1-week, mice displayed intact insulin-positive cells, which became more granulated at 2-weeks. At 3-weeks, staining for the insulin-positive cells were very dispersed, which demonstrated the timeline of xenograft rejection of NPIs [187]. In our experiments using NPI reconstitution, we conducted co-localization studies to observe the presence and infiltration of $CD45^+$ cells in relation to insulin-positive cells. Although our studies did not evaluate the difference between PBMCs from healthy donors or donors with type 1 diabetes, it may be worthwhile to investigate if such a difference exists. Further, it may be beneficial to investigate the early periods of NPI graft rejection as this may provide insight on the kinetics of NPI graft rejection using different PBMC doses.

Currently, low immunogenicity stem cells are being produced to address the problem of graft rejection, which could potentially allow the broad application of stem cell therapeutic

approaches. Such universal donor cells could potentially lower the dosage of required immunosuppressive medication to prevent graft rejection [189]. Though much research has been established in characterizing the *in vitro* response of these cells, *in vivo* experiments to study graft rejection have previously been incomplete by the limited timeframe from previous mouse models. In our experiments, we have demonstrated the feasibility of stem cell-derived islets to establish euglycemia in MHC class I/II double knockout mice. As well, we have characterized the ability of human PBMCs to reject these cells upon reconstitution. In future experiments, this model could serve to accommodate the usage of hypoimmunogenic stem cells that require a long timeframe for graft rejection studies. Although our experiments did not use a large sample of stem cell transplanted mice for reconstitution studies, we have still gained valuable insight on the general timeline of rejection of these cells. Further, this data could serve as the baseline for comparing the timeline of rejection using hypoimmunogenic stem cells.

It is important to mention the sex associated differences in PBMCs between males and females. It has previously been reported that mitochondrial activity in female PBMCs is significantly higher than those of males, and that ATP concentrations in PBMCs are also higher in females [240]. In our experiments, only PBMCs from female donors were used, which may have limited the breadth of our observations. Evaluating the differences in islet rejection between male and female PBMCs may be a worthwhile investigation to understand sex-related differences in human immune system function. Further, as previously mentioned, it is imperative to study the difference in PBMC activity between healthy donors and donors with type 1 diabetes. Future studies may yield further insights on the activity of the human immune system in patients with diabetes.

In our reconstitution experiments, it is important to mention that only male mice were utilized. This may have limited our observations as more data from female mice could benefit our conclusions. In the future, it is important to conduct experiments with both male and female mice to understand and characterize the intraspecies sex differences in relation to the kinetics of islet graft rejection.

To summarize the data, we observed rejection as indicated by hyperglycemia in mice transplanted with human islets as early as 5 days and as late as 18 days post-reconstitution. 53.8% of NPI transplanted and reconstituted mice displayed islet graft rejection, indicated by an absence of stimulated porcine insulin secretion at 6 weeks post-reconstitution. SC-β transplanted and reconstituted mice displayed rejection indicated by hyperglycemia at 21- and 23-days post-reconstitution. The data also shows that mice in all three conditions displayed co-localization of human immune cell infiltration in relation to insulin producing cells. While the work presented in this thesis can provide invaluable insight for future research, it is critical to understand how this study ties in with the past and current literature.

Past research has evaluated the immunogenicity and rejection of human, mouse, rat, and pig islets using a murine or, in some cases, a human immune system [178, 184, 270, 271]. The usage of a murine immune system was vital in allowing investigators to understand the apparent genotypic and immune composition differences between mice and humans [186]. This provided scholars with the knowledge to deviate from using a mouse immune system and towards using a human immune system. As well, this information provided researchers with a better understanding of the different immune mechanisms associated with graft rejection of islets in the context of either a human or murine immune system. Nonetheless, the usage of humanized mouse models is relatively new in the field of islet graft rejection compared to areas pertaining to

cancer research, indicating their unlimited potential in the study of islet graft rejection [179, 180]. Current literature demonstrates the significance of the new NSG-MHC I/II DKO mice and their ability to be used in islet graft rejection studies in the context of a human immune system, as these mice realistically imitate the key components of the human immune system [184]. In addition, these mice are not hampered in their potential by the development of GVHD, demonstrating their long-term applicability [184]. Future research can potentially use different techniques to engraft a human immune system in this strain of mice, such as using HSCs (hematopoietic stem cells), which can develop into a more complete human immune system [179, 180]. By using a more complete human immune system, researchers will be able to better apprehend immune components involved in graft rejection while not being hindered by the development of GVHD [179, 180]. In turn, this will allow researchers to better understand the rejection process of a variety of islet sources in the context of a human immune system.

In Appendix A, we conducted preliminary studies with stem cell-derived islets in Rag KO and NSG mice. Our objective was to first demonstrate that stem cell-derived islets could normalize diabetic Rag KO mice. Next, we transitioned to using NSG mice and demonstrated that streptozotocin-induced hyperglycemia could be reverse in this model through stem cell-derived islet transplantation. This eventually allowed us to merge into reconstitution studies using stem cell-derived islets in these same NSG mice. To demonstrate the *in vivo* maturation of stem cell-derived islets, we conducted intraperitoneal glucose tolerance tests over multiple time points. In one study, stem cell-derived islets transplanted in NSG mice displayed increased insulin secretion as indicated by insulin quantification between 4-weeks and 6-months post-transplantation [251]. Similarly, in our Rag KO mice transplanted with SC-β $\geq$30%, our data suggests that the stimulation index significantly increased between 8- and 12-weeks post-

transplantation. Our data suggests that it may be worth further evaluating insulin release over successive weeks. Our observations of the glucose clearance profile in Rag KO mice transplanted with either SC-β <30% or SC-β ≥30% displaying appealing results. Future research could benefit by demonstrating the difference in glucose clearance using a glucose challenge test in NSG mice transplanted with stem cells that co-express markers of mature β-cells to different degrees. Our data could also be strengthened by using a larger sample size of mice of both strains and sexes of mice. As well, it is important to further evaluate the differences in stem cell-derived islets that co-express mature β-cell markers to different degrees both *in vitro* and *in vivo*. Such an understanding will help future researchers optimize protocols to better differentiate stem cell-derived islets. As such, the ability to culture a homogeneous preparation of stem cells to a large degree may be closer within our reach.

In addition, although our research did not evaluate the teratogenicity of stem cell-derived islet preparations, it is essential for future researchers to focus on this topic in the context of an *in vivo* model. Future research on this subject could yield significant insight on controlling the teratogenicity of stem cell preparations, which could enhance patient results in clinical translations.

3-2 CONCLUSION:

The future of islet transplantation poses promising and exciting results. Islet transplantation has the potential to replace daily insulin injections for patients with type 1 diabetes to create a more physiological delivery of insulin, thereby reducing the micro and macrovascular complications [41]. For islet transplantation to be a more widespread treatment option, more suitable methods for controlling islet graft rejection must be used that eliminates or

reduced secondary complications associated with systemic immunosuppression. In addition, the usage of alternative β-cell sources could solve the problems associated with the usage of conventional human islets. For these advances to be realized in clinical settings, it is important that alternative β-cell sources are first studied in an established humanized mouse model that authentically mirrors the human immune system.

The objective of this study was to establish a reliable humanized mouse model that supports the survival of human, neonatal pig, and stem cell-derived islets, and display islet graft rejection upon reconstitution of a human immune system in the absence of GVHD. In this thesis, we have shown quantitative methods to evaluate islet graft rejection in both diabetic and naïve mice. Diabetic mice support the survival of the transplant and establish euglycemia. Upon reconstitution, the metabolic blood glucose profile displays a reversal to the diabetic state, indicating islet graft rejection. In naïve mice, we can see a functional decrease in insulin secretion during successive periods post-reconstitution. This indicates a functional decline in the ability of insulin producing cells to secrete insulin. As well, our qualitative observations reinforce and support our quantitative data. Our co-localization studies show the presence of human immune cell infiltration in the islet graft region, and hints towards the destruction of insulin producing cells. Histochemical observations provide a macroscopic overview of human immune cell infiltration in the graft region. Taken together, our observations indicate that continued establishment and study of this humanized mouse model is beneficial to enhancing our understanding of islet graft rejection.

It is my aspiration that the research presented in this thesis will provide an insight into further understanding of islet graft rejection. Further, I wish that this research will provide the

tools necessary to create a more suitable treatment option for patients with type 1 diabetes, in the near future.

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APPENDIX A

CELL COMPOSITION OF STEM CELL-DERIVED ISLETS AFFECTS

THE *IN VIVO* METABOLIC FUNCTION IN IMMUNODEFICIENT MICE

A-1 INTRODUCTION:

Stem cell-derived islet (SC-β) transplantation has promising potential as a future therapy. Clinical trials of cell replacement therapies conducted over the last decade have provided evidence of the potential to restore β-cell function in those with type 1 diabetes [1]. Though the usage of stem cell-derived islet sources is still in its infancy, much research has already shed light on the development and generation of pancreatic progenitor cells and induced pluripotent stem cells. Many groups have produced methods to reliably reproduce functional stem cell-derived islets that shadow the embryonic development of the pancreas [2 – 6]. Still, however, the optimization of SC-β sources remains an issue and much research needs to be conducted to augment the ability of SC-βs to enhance glucose stimulated insulin secretion while minimizing the potential teratogenic effects. In addition, the barriers posed from immunological rejection adds another layer of complexity that must be controlled [7].

Much research has already shown the potential for SC-βs to demonstrate glucose-responsive insulin secretion *in vitro* [5, 6]. However, the *in vivo* optimization of these islets has been less studied. For SC-β transplantation to become a more feasible treatment option in the future, it is important to study the glucose-responsive functionality and maturation of these islets *in vivo* while examining the teratogenic effects of different preparations [3]. In addition, it is vital to identify the ability of SC-βs to reverse hyperglycemia as well as the immunogenicity of these preparations. However, before the immunogenicity of SC-βs can be characterized in the context of a human immune system, it is important to test the ability of these stem cells to establish euglycemia in an already established murine model, as well as a murine model that will be used for future reconstitution studies. To do this, it is important to identify the animal sources that will be examined in this preliminary study.

B6.1297S-Rag1^{tm1Mom} mice (Rag) are an immunodeficient strain of mice that contain the

recombinant activating gene (RAG) knockout, which inhibits the functional development of T-

and B-lymphocytes in this strain [8]. This mouse strain has been extensively used in many

previous transplantation studies using different islet sources and have shown potential to

establish euglycemia [9, 10]. Therefore, these mice provide a good starting point for conducting

preliminary transplantation studies involving the usage of SC-βs to indicate the ability of the

islets to establish euglycemia. However, this murine strain is limited beyond simple euglycemic

studies and cannot become reconstituted with a human immune system due to the presence of

mouse natural killer (NK) cells that destroy human immune cells upon injection [11]. This is

mainly due to the presence of the IL2 Receptor that is present in this specific strain of mice [12].

To combat this limitation, it is important to identify a mouse strain that can be transplanted with

SC-βs and establish euglycemia as well as become reconstituted with a human immune system.

A specific strain of non-obese diabetic (NOD) mice termed non-obese diabetic SCID Gamma

(NSG) mice that contain the MHC class I and II knockout (NSG-MHC I/II DKO) and are

deficient in the *IL2* receptor can be used for the purpose of transplantation and reconstitution.

The lack of functional T-, B-, and NK cells in this strain allows them to become reconstituted

with a human immune system [11, 13]. However, before reconstitution studies can be conducted,

it is important to characterize the ability SC-βs to establish euglycemia as well as the *in vivo*

functionality of in both strains of mice.

The objective of this preliminary research is to investigate the metabolic difference in

experimental transplantation of SC-βs in mice, based on different cell compositions of SC-βs.

More specifically, SC-βs greater than and less than 30% double positive for C-peptide and

NKX6.1 using flow cytometry were compared in two different strains of mice, NSG MHC I/II

DKO (simplified as NSG) and Rag mice. In addition, the cells were investigated for their potential to release insulin *in vivo.* The rationale for transplanting SC-βs into Rag mice first and transitioning into NSG mice was to initially test the function of SC-βs in Rag mice, which are an already established mouse model, and then transition to using NSG mice as they possess the potential to become reconstituted with a human immune system.

A-2 EXPERIMENTAL DESIGN:

A-2.1 Mice:

Six-week-old male NOD.SCID gamma $(K^BD^B)^{null}$ $(IA)^{null}$ mice (NSH-MHC I/II DKO or NSG) and six-to-eight-week-old male and female B6.1297S-*Rag1*^{tm1Mom}/J (Rag) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used as recipients for stem-cell derived islet (SC-β) transplantation. All mice were housed in a pathogen-free, climatized environment at the Health Sciences Laboratory Animal Services Facility of the University of Alberta. All animals were fed standard laboratory food and given water containing Novotrimol *ad libitum*. Animal use was in accordance with the Canadian Council on Animal Care and approved by the institutional animal ethics committee at the University of Alberta, Edmonton AB, Canada (AUP00000278, AUP00002977).

Diabetes Induction in Mice:

All mice used for the purpose of transplantation were made diabetic through an intraperitoneal injection of streptozotocin (STZ) at 185 mg/kg (male) or 180 mg/kg (female) (Sigma, St. Louis, MO, USA). Mice were confirmed to be diabetic when the blood glucose level was ≥17.7 mmol/L for two consecutive days. Mice that did not achieve a blood glucose level of

≥17.7 mmol/L were not used for transplantation. Once diabetes was confirmed, each mouse received a LinBit (Linshin, Toronto, Canada) implanted subcutaneously. LinBits were replaced during the time transplantation and subsequently removed at 4 weeks post-transplantation. Blood glucose and weights of mice were monitored once weekly prior to transplantation.

A-2.2 Stem Cell-Derived Islet Transplantation:

Stem cell-derived islets (SC-βs) (University of Toronto, Toronto, Canada) were kindly provided by Dr. Nostro's lab at the University of Toronto. Cells were differentiated in culture for 20-26 days based on the protocol established by Hogrebe et al. [231]. At 19-20 days, the media was changed to a solution of MCDB-131 (Wisent Bio Products, Saint-Jean Baptist, QC, CA), supplemented with 7.5% sodium bicarbonate (Gibco), Glutamine, D-Glucose (MilliporeSigma), Zinc Sulfate (MilliporeSigma), Fatty acid free Bovine serum albumin (FAF-BSA, ProLiant Biologicals, Ankeny, IA, USA) and the cells were shipped overnight in a 50 mL conical tube containing this media. Prior to shipment, cells were assessed for markers of mature β-cell function co-expressing NKX6.1 and C-peptide using flow cytometry. Prior to experiment #8, islets were not incubated and were immediately aliquoted for transplantation on the same day. Following an alternative protocol for experiment #9 and onwards, islets were gravity settled and the pellet was removed and put into a 150 mm non-coated petri dish. The media was centrifuged at 1200 rpm for two minutes to remove dead cell debris and this media was then used to culture the islets overnight at 37°C with 5% CO_2 and 95% oxygen. The following morning, approximately ~2.5 to 6.0 x 10^6 cells were aliquoted into Eppendorf tubes in preparation for transplantation. All studies were approved by the Human Research Ethics Board at the University of Alberta (Pro00092479).

Transplanted animals were segregated into two groups: 1) Mice transplanted with SC-β less than 30% double positive for NKX6.1 and C-peptide (SC-β <30%) (Table 7), and 2) Mice transplanted with SC-β greater than or equal to 30% double positive for NKX6.1 and C-peptide (SC-β ≥30%) (Table 8).

Table 7: Summary of all experiments where mice were transplanted with SC-β <30%. Top sections of the table display characteristics of stem cells, including the stem cell ID, percent double positive for NKX6.1 and C-peptide and whether an overnight culture was performed upon arrival at the lab. Cells were counted using a hemocytometer for clarification. Bottom sections of the table display transplantation of mice, including how many cells were transplanted per mouse in each experiment, as well as the euglycemic ratio. A technical error occurred in experiment 12 where 3 mice were supposed to be transplanted initially, but the stem cells were lost during the process.

Experiment #:	1	2	3	9	10	12	13	14
Stem Cell-Derived Islet Characteristics								
Stem cell ID:	CNIL3, D26 11/15/2020	ASIL4, D23 02/08/2021	ASIL6, D23, 02/16/2021	ASIL11, D24 05/10/2021	ADIL12, D23 05/18/2021	ASIL17, D22 07/20/2021	ASIL18, D24 08/04/2021	ASIL19, D22 08/17/2021
% Double Positive	20.8%	17.5%	14.1%	26.9%	22.2%	7.71%	24.9%	28.3%
Overnight Culture (Y/N):	No	No	No	Yes	Yes	Yes	Yes	Yes
Cell Count in vial (x 10^6 cells):	12.80	6.00	27.00	7.00	10.56	12.30	20.40	30.40
Cell Count (Hemocytometer): (x 10^6 cells):	NA	NA	NA	4.03	9.96	8.00	22.27	30.50
Transplant								
Mice (n=) & Strain:	n=3 NSG n=2 Rag	n=2 Rag	n=5 Rag	n=2 Rag	n=2 Rag	n=2 NSG	n=2 NSG n=2 Rag	n=5 NSG
Sex (M/F):	Male	Male	Male	Female	Female	Male	Male	Male
Date of Tx:	November 17, 2021	February 9, 2021	February 17, 2021	May 12, 2021	May 20, 2021	July 22, 2021	August 6, 2021	August 19, 2021
Mortalities:	None	None	None	None	1 death	1 death	None	2 deaths
LinBit removal (days post-transplant):	27	31	30	26	25	28	24	25
Cells/Mouse:	~2.5 x 10^6	~3 x 10^6	~5x10^6	~3.5 x 10^6	~5 x 10^6	~4 x 10^6	~5 x 10^6	~6 x 10^6 cells
Euglycemic ratio:	0/3 NSG 0/2 Rag	0/2	0/5	0/2	1/1	0/1	1/2 NSG 2/2 Rag	3/3

Table 8: Summary of all experiments where mice were transplanted with SC-β ≥30%. Top sections of the table display stem cell characteristics such as the stem cell ID and the percentage of stem cells that were double positive. The stem cell ID also includes the total period of how long the cells were cultured for (i.e., D20 indicates that the cells were cultured for 20 days prior to shipment). The bottom sections of the table display the transplantation characteristics including how many days post-transplantation the LinBits were removed in each experiment as well as the euglycemic ratio at the end of the time period.

Experiment #:	5	6	7	8	11	15
Stem Cell-Derived Islet Characteristics						
Stem cell ID:	FSIL6, D20, 03/11/2021	FSIL7, D21 03/17/2021	FSIL8, D22 04/07/2021	ASIL10, D22 04/21/2021	ASIL13, D23 06/01/2021	FSIL16, D24 11/17/2021
% Double Positive:	45.8%	37.0%	55.1%	47.5%	35.8%	60.3%
Overnight Culture (Y/N):	No	No	No	Yes	Yes	Yes
Cell Count in vial (x 10^6 cells)::	16.50	21.00	12.87	12.60	6.23	15.00
Cell Count (Hemocytometer) (x 10^6 cells)::	8.16	18.23	10.40	9.05	NA (away)	13.47
Transplantation						
Mice (n=) & Strain:	n=4 Rag	n=4 Rag	n=3 Rag	n=3 Rag	n=2 Rag	n=3 NSG
Sex (M/F):	Male	Male	Female	Female	Male	Male
Date of Tx:	March 11, 2021	March 18, 2021	April 8, 2021	April 23, 2021	June 3, 2021	November 19, 2021
Mortalities:	None	None	1 death	None	None	1 death
LinBit removal (days post-transplant):	25	26	26	24	25	24
Cells/Mouse:	~4 x 10^6	~5.25 x 10^6	~4 x 10^6	~4 x 10^6	~3 x 10^6	~5 x 10^6
Euglycemic ratio:	3/4	3/4	2/2	3/3	1/2	2/2

Mice undergoing transplantation were anaesthetized through isoflurane inhalation (1.5%) and the left flank was shaved and disinfected using 70% ethanol. The skin was swabbed three times with chlorhexidine (Thermo Fisher Scientific) using a sterile gauze. Mice were subcutaneously administered buprenorphine (0.05-0.1 mg/kg) (Western Drug Distribution Center Ltd., Edmonton, AB, CA) using a 27-guage needle and 1 mL syringe. Afterwards, the left kidney was localized through the skin of the mouse and an incision was made in the skin and muscle layer near the shaved flank area using iris scissors. The kidney was uncovered from the perirenal space using a sterile cotton swab and a small incision was made in the kidney capsule (KC) using a 27-gauge needle. Aliquots of ~2.5 to 6.0×10^6 SC-βs were aspirated into polyethylene (PE-90) tubing, pelleted by centrifugation, and then gently transferred under the KC with the aid of a micromanipulator syringe. Following transplantation of the islets, the KC was cauterized using a cautery pen to prevent the leakage of transplanted cells. The kidney was then placed back into the perirenal space and the incision in the muscle layer was sutured using a 5-0 Vicryl suture thickness and the skin was stapled. During the time of transplant, LinBits previously transplanted subcutaneously were replaced and subsequently removed 4-weeks post-transplantation. Mice were then placed in a warm cage and closely monitored for recovery. All transplanted mice were closely monitored for the presence of low blood glucose ($\leq$ 4 mmol/L) and subsequently administered 100-300 µL of D-glucose via IP injection.

A-2.3 Metabolic Follow-up:

Blood Glucose and Weight Measurements:

Non-fasting blood glucose and weight measurements of mice transplanted with SC-βs were obtained from the tail vein (OneTouch UltraMini glucose meter) once per week. Mice were considered euglycemic when the blood glucose level was ≤11.1 mmol/L for one consecutive week. After removal of the LinBit at 4-weeks post-transplant, alternative week fasting blood glucose levels were measured. To obtain fasting blood glucose values, mice were fasted overnight for 12-hours, and blood glucose was again measured from the tail vein. Fasting blood glucose values were taken as an indirect measure to assess the degree of SC-β functionality. After 20-weeks, mice that maintained euglycemia underwent a survival nephrectomy where the islet bearing kidneys were removed. The reversal of blood glucose of mice into hyperglycemia confirmed that the euglycemic status was due to insulin release from the transplanted SC-βs. Mice are considered diabetic when the blood glucose level was ≥17.7 mmol/L.

Stimulated Graft Insulin Secretion:

An intraperitoneal glucose tolerance test (IPGTT) was used to indicate the responsiveness of SC-βs to fasting and glucose stimulated conditions, measured as the degree of insulin secretion. To determine the degree of insulin secretion and to assess the *in* vivo maturation of islets, human insulin was analyzed during an 8-, 12-, and 20-week IPGTT. In addition, to determine the glucose clearance profiles of mice, the blood glucose profile was plotted during a 20-week intraperitoneal glucose challenge test. After a 12-hour fast, blood samples were obtained from the tail vein at 0 minutes. Following, D-glucose (3 mg/g) was administered IP, and blood was again collected from the tail vein at 60 minutes. Concurrently, blood glucose

measurements were taken from the tail vein at 0 and 60 minutes during the 8- and 12-week

IPGTT, and at 0, 15, 30, 60 and 90 minutes during the intraperitoneal challenge test at 20-weeks.

Immediately, samples were placed on ice and centrifuged at 10,000 rpm at 4°C for 10 minutes.

Serum samples were collected to detect the presence of graft-specific human insulin.

Fasting (0 minute) and stimulated (60 minute) human insulin levels were measured using

ALPCO enzyme-linked immunosorbent assay (ELISA) (ALPCO, Salem, NH, USA). This assay

detects human insulin at 100% and cross-reacts with porcine insulin at 175% but does not cross-

react with mouse or rat insulin (0%).

A-2.4 Immunohistochemical Graft Characterization:

Graft-bearing kidneys were collected and fixed in 10% paraformaldehyde (BDH

Laboratory Supplies) and then embedded in paraffin. Tissue sections of 5 μm thickness were

sliced. Tissue sections were rehydrated and subject to heat-mediated antigen retrieval using

citrate buffer (pH 5.50). Slides were quenched in a mixture of methanol and hydrogen peroxide

for 6 minutes to remove endogenous peroxidase and the quenching reaction was immediately

stopped by placing the slides in water. Afterwards, slides were incubated for 60 minutes with

20% normal goat serum (NGS, Jackson for ImmunoResearch Laboratories Inc), and

subsequently incubated with rabbit anti-human C-peptide primary antibody (1:500, Bio-Rad

Laboratories, Hercules, CA, USA) for 60 minutes. Following, an incubation was performed

when the slides were applied with biotinylated goat anti-rabbit secondary antibody (1:200,

Jackson ImmunoResearch Laboratories Inc) for 30 minutes. Avidin-biotin complex (ABC,

Vector Laboratories, Burlingame, CA, USA) was applied to the tissue sections and incubated for

40 minutes, followed by application of 3,3-diaminobenzidine (DAB, BioLegend, San Diego, CA,

USA) to produce a brown color for positive cells. Subsequently, the reaction was immediately stopped by placing the slides in water, and the sections were counterstained with Harris' hematoxylin and eosin (H&E) and cover slipped. After drying, slides were visualized using the Nikon ECLIPSE TS2 inverted microscope (Nikon, Melville, NY, USA).

A-2.5 Statistical Analysis:

Data are represented as mean ± standard error of the mean (SEM). Differences between the groups were analyzed using one sample, two-way t-test, or a one-way ANOVA with Tukey's post-hoc test for the analysis of variances for multiple comparisons between groups. All comparisons were performed using a 95% confidence interval and a p-value of $*p<0.05$ was considered statistically significant. Statistical analysis was conducted using GraphPad Prism software (GraphPad Software, La Jolla, Ca, USA).

A-3 RESULTS:

A-3.1 Metabolic Follow-up of Stem Cell-Derived Islet Transplanted Mice:

Non-fasting blood glucose and weights of mice were monitored weekly. Overall, 23 mice survived the transplant with SC-β <30%. 9 mice were of the NSG strain, of which all were males, whereas 14 were Rag mice. 3 Rag mice were female, and 11 were males. Another 17 mice transplanted with SC-β ≥30% survived. There were 2 NSG mice, both of which were males, and 15 Rag mice, of which 5 were females and 10 were males.

21.4% of Rag mice transplanted with SC-β <30% achieved complete euglycemia (Figure 13A) compared to 80% of Rag mice transplanted with SC-β ≥30% (Figure 13B). 44.4% of NSG mice transplanted with SC-β <30% achieved complete euglycemia (Figure 13C) compared to
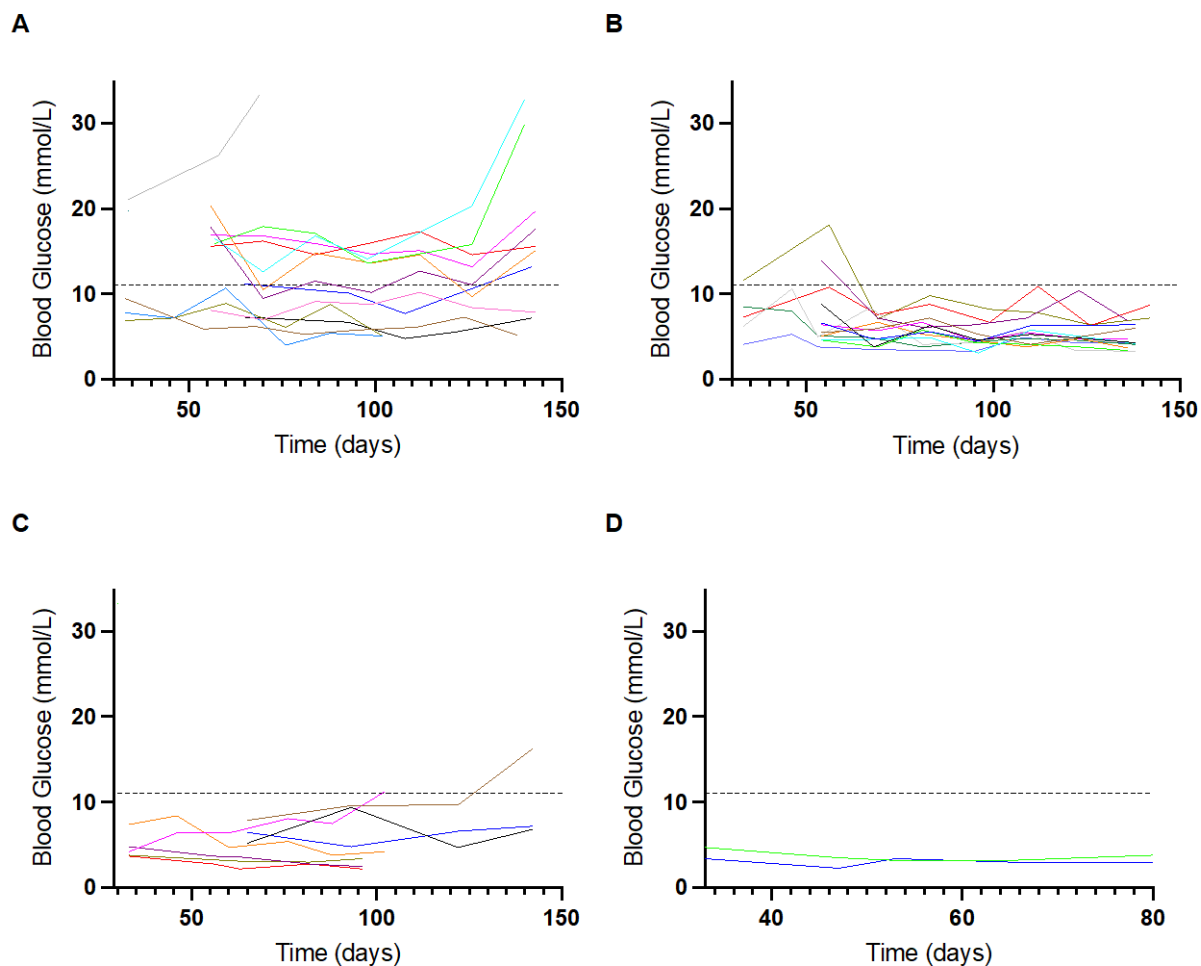
100% of NSG mice transplanted with SC-β ≥30% (Figure 13D). Of the mice transplanted with SC-β ≥30%, 46.7% of Rag mice displayed euglycemia at 50 days, which increased to 80% by 140 days (Figure 13B), and none of the NSG mice transplanted with SC-β ≥30% displayed euglycemia at 45 days but this increased to 100% at 80 days (Figure 13D).

Of the Rag mice transplanted with SC-β <30%, 3 mice underwent survival nephrectomy and displayed successful reversal to hyperglycemia, indicating that the euglycemic level was due to the transplanted SC-βs (Figure 13A). Of the Rag mice transplanted with SC-β ≥30%, 12 mice underwent survival nephrectomy, and 83.3% displayed a reversal to the hyperglycemic state (Figure 13B). None of the NSG mice underwent survival nephrectomies as they were either already hyperglycemic or used for reconstitution purposes.

Following removal of subcutaneously transplanted LinBits after 4-weeks, alternative week fasting blood glucose measurements were taken as an indication of metabolic regulation and function of SC-βs in mice under fasted conditions. Based on the analyzed mice, 75% of Rag mice transplanted with SC-β <30% maintained euglycemic fasting blood glucose levels throughout the experiment (Figure 14A), compared to 100% of Rag mice transplanted with SC-β ≥30% (Figure 14B). 88.8% of NSG mice transplanted with SC-β <30% maintained euglycemic fasting blood glucose levels throughout the experiment (Figure 14C), compared to 100% of NSG mice transplanted with SC-β <30% (Figure 14D).
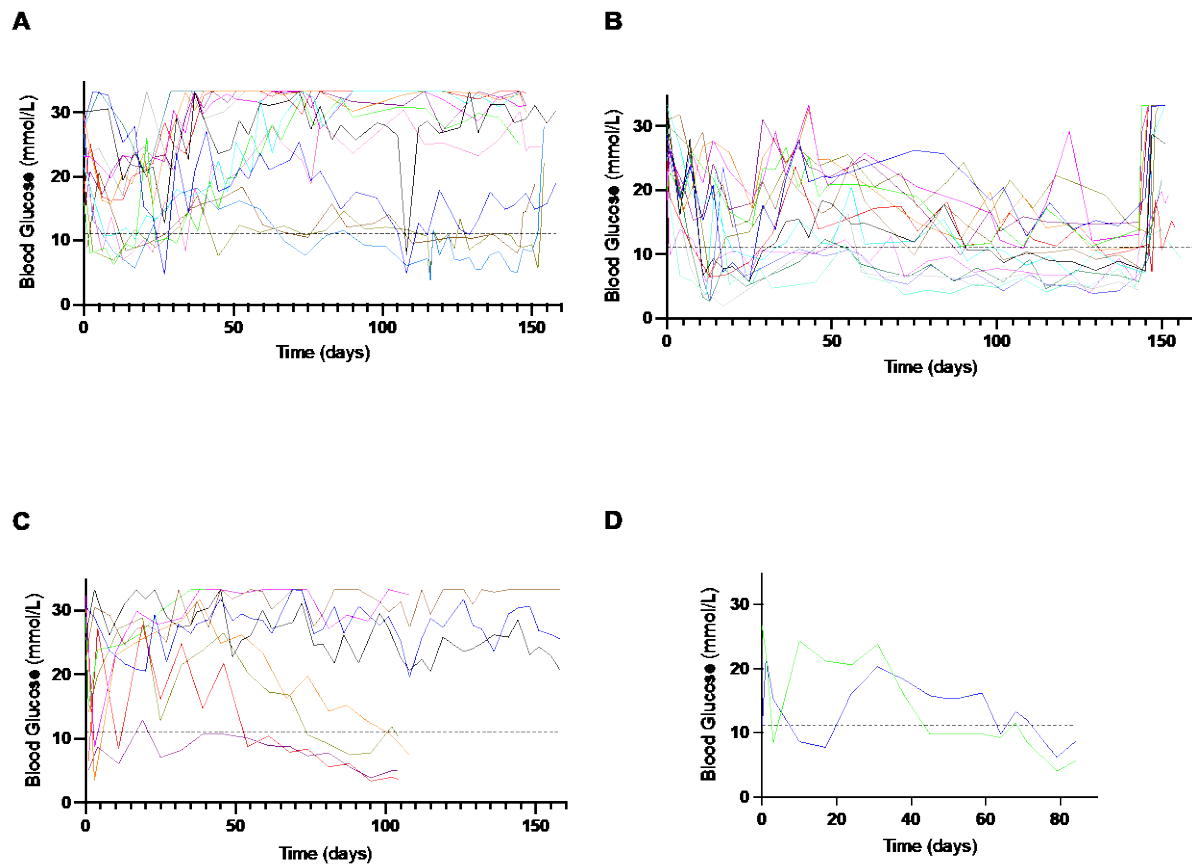
Figure 13: Non-fasting blood glucose levels of Rag and NSG mice transplanted with SC-β. **A)** Rag mice transplanted with SC-β <30% double positive for NKX6.1 and C-peptide. n=14. **B)** Rag mice transplanted with SC-β ≥30% double positive for NKX6.1 and C-peptide. n=15. **C)** NSG mice transplanted with SC-β <30% double positive for NKX6.1 and C-peptide. n=9. **D)** NSG mice transplanted with SC-β ≥30% double positive for NKX6.1 and C-peptide. n=2.

Figure 14: Non-fasting blood glucose levels of Rag and NSG mice transplanted with SC-β. **A)** Rag mice transplanted with SC-β <30% double positive for NKX6.1 and C-peptide. 13 mice were analyzed. In one mouse, only one fasting blood glucose value was obtained, and the mice was euthanized due to low level of double positive transplanted cells. This mouse has not been included in these observations. **B)** Rag mice transplanted with SC-β ≥30% double positive for NKX6.1 and C-peptide. 15 mice were analyzed. **C)** NSG mice transplanted with SC-β <30% double positive for NKX6.1 and C-peptide. 8 mice were analyzed. In one mouse, only one fasting blood glucose value was obtained, and the mouse was euthanized due to poor blood glucose regulation. This mouse has not been included in these observations. **D)** NSG mice transplanted with SC-β ≥30% double positive for NKX6.1 and C-peptide. 2 mice were analyzed.

Stimulated insulin secretion was analyzed in mice transplanted with either SC-β <30% or SC-β ≥30%, at 8-, 12-, and 20-weeks post-transplant. During the initial experiments conducted at the beginning of the study, mice that were hyperglycemic or mice that were transplanted with a low level of double positive stem cells still underwent glucose tolerance tests. However, for experiments conducted near the end of the study period, mice that maintained a high blood glucose level or mice that were transplanted with a low percentage of double positive stem cells did not undergo glucose tolerance tests.

ELISA was used analyze human insulin secretion in plasma collected at 0 and 60 minutes over the duration of 8-, 12-, and 20-weeks. During analysis, insulin that was below the lower limit of detection (i.e., non-detectable) on the ELISA standard curve was assigned the lowest value on a standard curve (2.78 pmol/L). All values were taken to two decimal points and outlier values were removed from analysis.

For mice that were transplanted with SC-β <30%, Rag mice and NSG mice were separately analyzed, and the SI was calculated. According to Figure 15A, there were no statistically significant differences in Rag mice transplanted with SC-β <30% ($p>0.05$). Similarly, the SI did not show any statistical significance between the different time points in these mice (Figure 15B). However, for NSG mice transplanted with SC-β <30%, there was a statistically significant difference between the basal and stimulated insulin levels at 12-weeks (*$p<0.05$) (Figure 15C). However, there was no difference between the SIs at different time points (Figure 15D).

In the same manner, for mice that were transplanted with SC-β ≥30%, Rag mice and NSG mice were separately analyzed, and the SI was calculated. Results showed that Rag mice displayed a statistically significant differences between the basal and stimulated insulin secretion

at 8-weeks (*p<0.05), 12-weeks (**p<0.01), and 20-weeks (***p<0.001) (Figure 16A). When looking at the SI, there was a statistically significant difference between the 8- and 12-week SI (*p<0.05) but not between the 8- and 20-week SI (p>0.05) or between the 12- and 20-week SI (p>0.05) (Figure 16B). For NSG mice, it is important to keep in mind that there was only one cohort used in this experiment and the data may be limited. Furthermore, this cohort did not undergo a 20-week IPGTT as these mice were eventually used for the purpose of reconstitution. Based on Figure 16C, NSG mice transplanted with SC-β ≥30% displayed insulin secretion at basal and stimulated levels. The SI displayed a slight increase in value between 8- and 12-weeks (Figure 16D).

At 20-weeks post-transplantation, mice transplanted with SC-β <30% or SC-β ≥30% underwent a glucose challenge test to indicate the degree of glucose clearance. NSG mice were not analyzed due to only a low number of these mice that underwent the 20-week glucose challenge test.

Glucose clearance profiles in Rag mice transplanted with SC-β <30% or SC-β ≥30% were compared. Results demonstrate that Rag mice transplanted with SC-β ≥30% displayed accelerated glucose clearance in comparison with Rag mice transplanted with SC-β <30% (Figure 17A). Analysis of the area under the curve (AUC) demonstrated that there was a significant difference in glucose excursion in Rag mice transplanted with SC-β ≥30% and those transplanted with SC-β <30% (****p<0.0001) (Figure 17B).
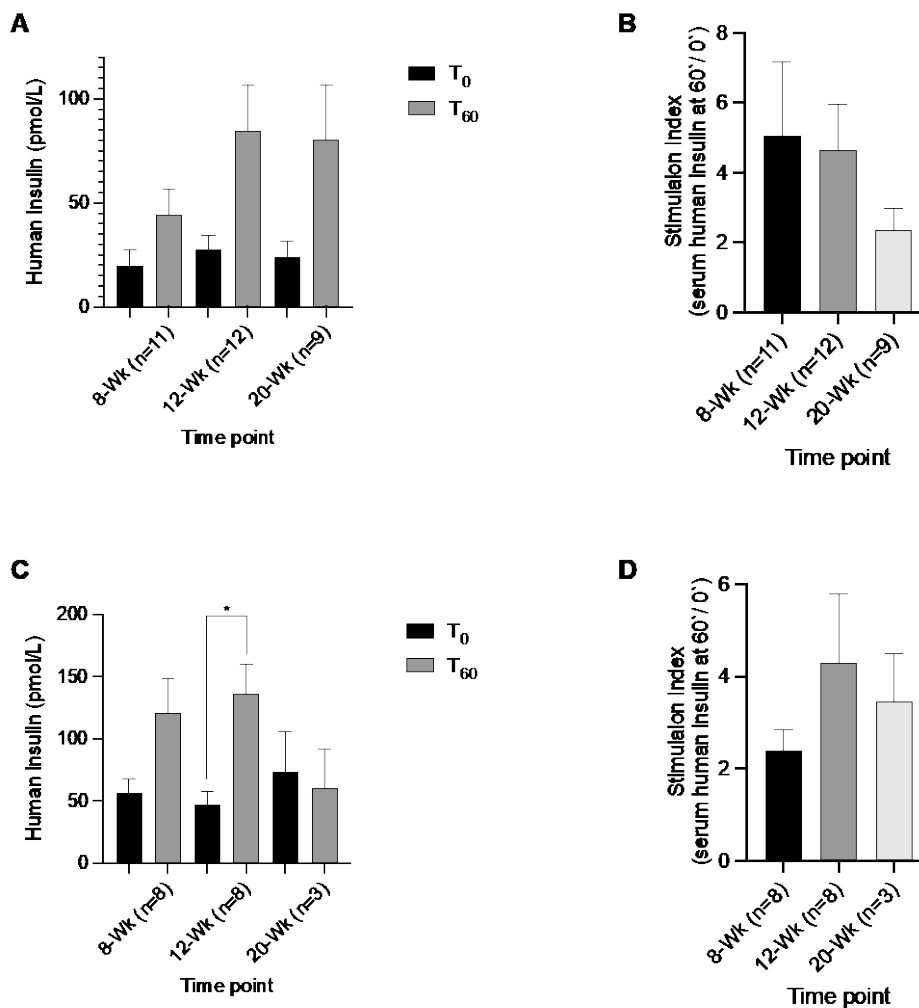
Figure 15: A) Human insulin stimulated secretion of Rag mice transplanted with SC-β <30%. No significant differences were observed between any time points. At 8-weeks, 1 Rag mouse in experiment 1 was removed from analysis due to technical error. At 20-weeks, 1 Rag mouse from experiment 3 was removed from analysis due to the presence of an outlier value. **B)** Stimulation index of Rag mice transplanted with SC-β <30%. No significant differences were observed. **C)** Human insulin stimulated secretion of NSG mice transplanted with SC-β <30%. A significant difference was observed in insulin secretion at the 12-week timepoint between basal (time 0) and stimulated (time 60) insulin secretion. **D)** Stimulation index of NSG mice transplanted with SC-β <30%. No significant differences were observed between the time points. (ns $p > 0.05$, $^{*}p < 0.05$).
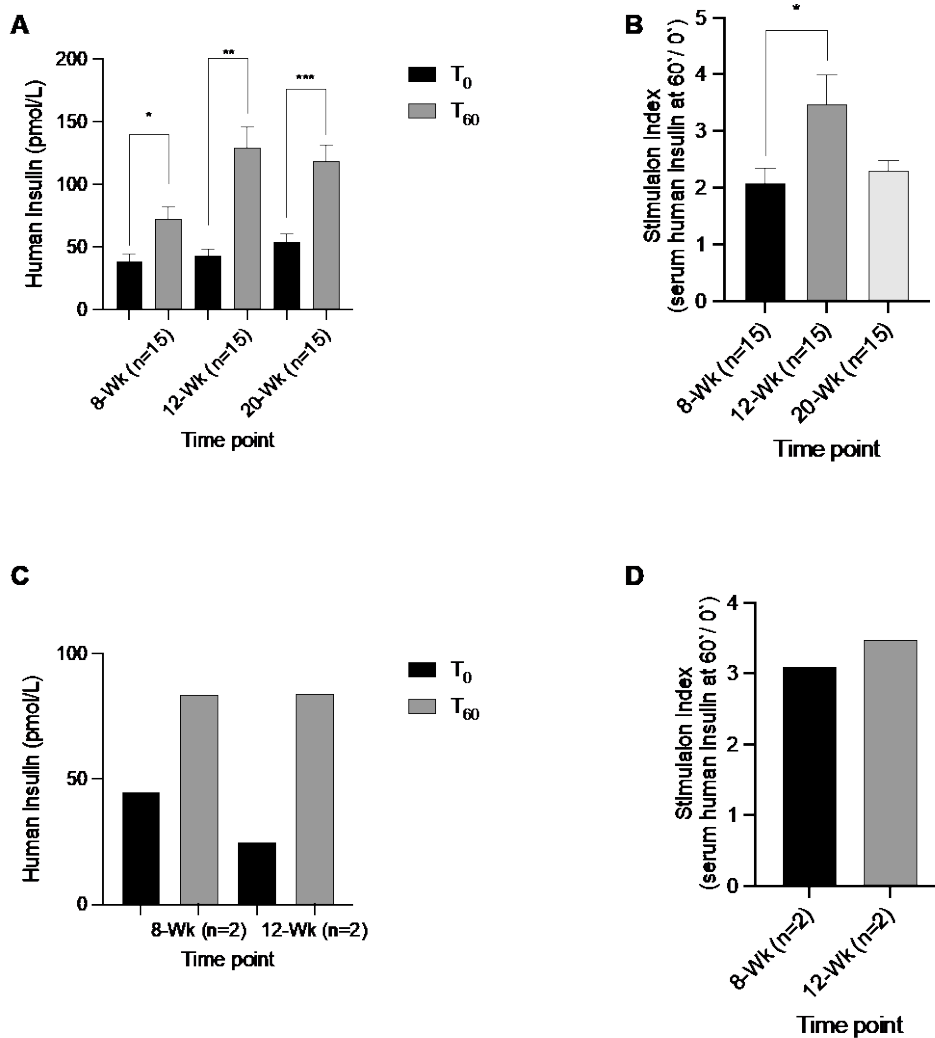
Figure 16: A) Human insulin stimulated secretion of Rag mice transplanted with SC-β ≥30%. Significant differences were observed between the basal (time 0) and stimulated (time 60) insulin secretion at 8-weeks, 12-weeks, and 20-weeks. **B)** Stimulation index of Rag mice transplanted with SC-β ≥30%. A significant difference was observed between the 8- and 12-week time points. **C)** Human insulin stimulated secretion of NSG mice transplanted with SC-β ≥30%. **D)** Stimulation index of NSG mice transplanted with SC-β ≥30%. (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$).
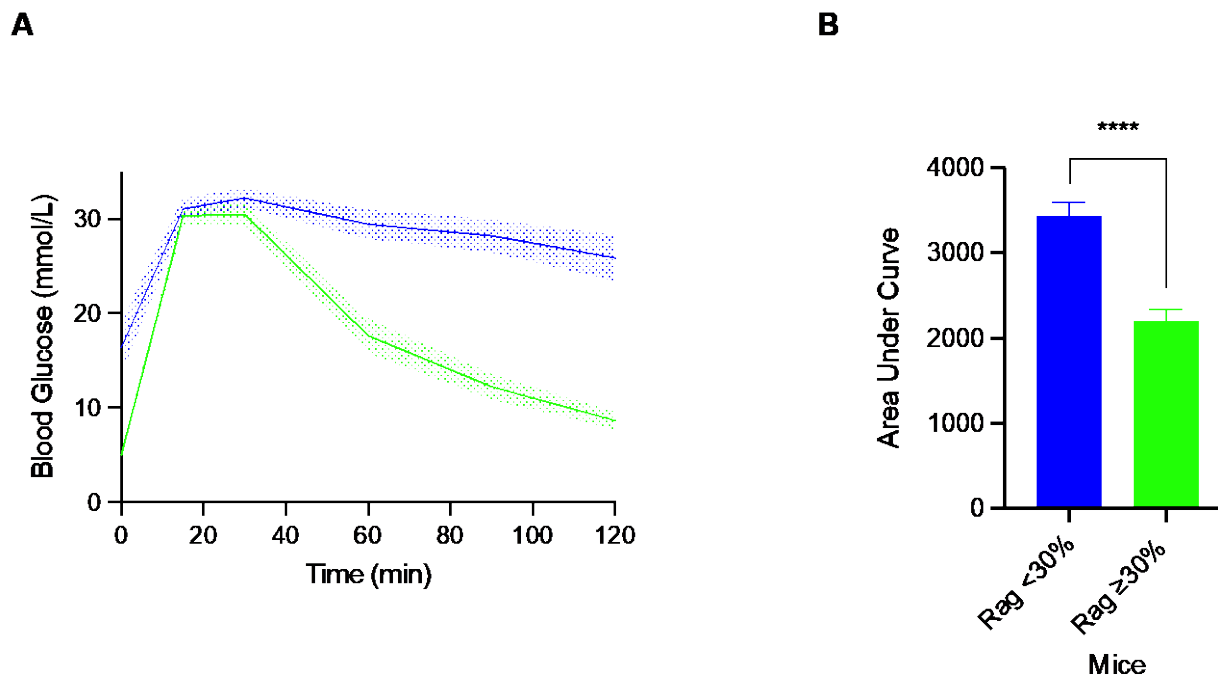
Figure 17: Glucose clearance profiles of mice during a 20-week post-transplant intraperitoneal glucose challenge test. **A)** Glucose challenge test for Rag mice transplanted either with SC-β <30% (n=10) or with SC-β ≥30% (n=15). Mean values are taken, and shaded areas represent the SEM. Blue indicates mice transplanted with SC-β <30% whereas green indicates SC-β ≥30%. **B)** Area under the curve values for mice transplanted either with SC-β <30% (n=10) or SC-β ≥30% (n=15). Mean values were taken, and error bars represent SEM. Blue indicates mice transplanted with SC-β <30% whereas green indicates SC-β ≥30%. (B; ****p<0.0001).

A-3.2 Graft Characterization Results:

Immunohistochemistry staining of tissue sections displayed positive staining for human C-peptide in both groups (Figure 18A, B) at 10X magnification. Staining also displayed the morphological characteristics of the islet graft. Images display representative graft sections and staining that was seen in both groups. Mice in both groups were euthanized at 21 weeks post-transplant. There was no visual difference in the staining between the two groups. Although quantification of the positive staining section was not performed, this may provide further information regarding the maturation of cells and the differences between SC-β <30% and SC-β ≥30%.
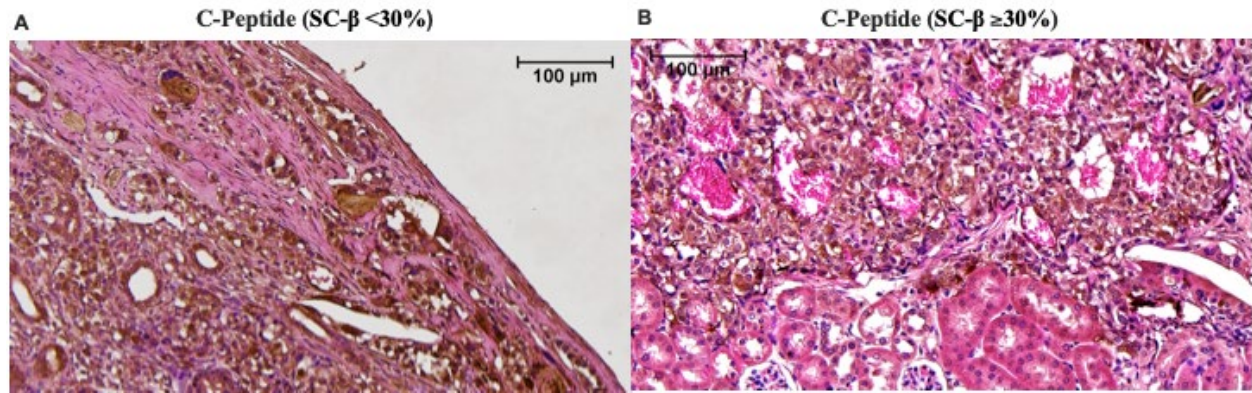
Figure 18: Immunohistochemistry staining of islet grafts from Rag mice in experiment 2 **(A)** and experiment 6 (**B**). Both mice were euthanized at 21 weeks post-transplant. Positive staining for C-peptide is indicated by brown color. No visual differences are observed between C-peptide content in mice transplanted with SC-β <30% and SC-β ≥30%. Quantification of positive C-peptide content was not performed. Images are displayed at 10X magnification.

A-4 DISCUSSION:

The usage of stem cell transplantation as an alternative treatment option to conventional human islet use poses very promising results as a future therapy. The usage of stem cells contains many advantages over human islet usage. Namely, by creating an unlimited cell supply with the potential for enhancements through genetic modification, SC-βs can overcome the limitations of human islet use, mainly the shortage of human donors and possibly reduce the need for chronic immunosuppressive medications [1]. However, before SC-β transplantation becomes the norm for the treatment of patients with type 1 diabetes, much experimental research must be conducted to establish the efficacy of using this source. By using a wide variety of preparations with a collection of cell markers of different positivity rates, researchers will gain a more comprehensive understanding of the optimal cell preparations necessary for clinical use. Furthermore, as research progresses, it is imperative that an established protocol is developed that can produce a consistent, homogeneous preparation of SC-βs in mass quantities that are standardized for optimal results in the clinic [14]. Prior to achieving this final step, researchers must first evaluate the efficacy of SC-βs in reversing diabetes as well as minimizing the teratogenic effects of such cells.

Previous research has also looked at the transplantation of SC-βs in mice. Maxwell et al. [15] suggests that the functionality and maturation of SC-βs increase post-transplantation in mice. Our research is in line with these observations, as we have observed that 7/15 Rag mice transplanted with SC-β ≥30% achieved euglycemia within 50 days, which increased to 12/15 Rag mice at 140 days. Furthermore, at 45 days post-transplantation, 0/2 NSG mice were euglycemic, compared to 80 days where they both achieved euglycemia. Although the research conducted by Maxwell et al. [15] evaluated the usage of different amounts of SC-βs for

achieving euglycemia in NSG mice (0.75, 2.0, and 5.0 million cells), our research did not evaluate the statistics of different amounts of islets used. Nonetheless, Maxwell et al. [15] suggest that they were able to achieve euglycemia in diabetic NSG mice transplanted with as little as 2.0 million stem cell-derived islets [15]. In our experiments, it is important to acknowledge that we transplanted at least 2 million SC-βs in every mouse, which is in accordance with the level used by previous research [15, 16].

In another research article, Augsornworawat et al. [16] discovered that SC-βs transplantation increases islet-amyloid polypeptide (IAPP) as well as additional changes in the transcriptome of genes upon transplantation that render it close to the resemblance of adult β-cells. The authors also noted that they observed an increase in functionality of SC-βs post-transplantation. This was observed when analyzing GTT data conducted on NSG mice at 4-weeks and 6-months post-transplantation, analyzing for human c-peptide secretion [16]. Similarly, our study observed an increase in human insulin secretion in Rag mice transplanted with SC-β ≥30% between the 8- and 12-week stimulation index, suggesting maturation of stem cells *in vivo*, although we did not analyze transcriptome increase through RNA-sequencing. Further, although we did not statistically evaluate the stimulated insulin secretion of NSG mice transplanted with SC-β ≥30%, using a larger cohort of NSG mice in the future may provide beneficial results.

Based on our other observations, it may be worth investigating the glucose clearance profiles of NSG mice transplanted with either SC-β <30% or SC-β ≥30%. Such observations may yield significant insight on further optimization of stem cell differentiation protocols. By comparing the *in vitro* observations to the *in vivo* results, researchers may gain a better understanding of further optimizing stem cell differentiation protocols.

In our experiments with stem cells, not all mice displayed a return to the hyperglycemic state after a survival nephrectomy. This may be due to pancreatic regeneration that could have possibly occurred during the timeline of the experiment, through natural regenerative responses [18].

Many studies have previously evaluated the markers that signify the maturation in populations of stem cell-derived islets [3 – 6 ,17]. This is a common method to evaluate the differentiation and purity of SC-βs, which can be compared to markers present in cadaveric human islets [6]. However, no study has yet evaluated the differences in using SC-β <30% or SC-β ≥30% double positive for NKX6.1 and C-peptide in animal models. Therefore, this study provides an initial understanding of the necessary cell conditions required to establish euglycemia in animal models. Further, an enhanced understanding of the behavior of SC-βs in mice will provide researchers with a better understanding in humans, which can assist in transitioning novel therapies to widespread clinical translation.

Although this preliminary study provides useful information for future studies, there are several limitations that must be addressed to create more conclusive results. Primarily, the number of cells transplanted under the KC of mice must be controlled. This would control some of the variability in data and provide more convincing results. In addition, using a larger sample size for both Rag and NSG mice and including both sexes for NSG mice would provide more useful information. It is important to evaluate the interspecies sexual differences between animals, and our data is limited due to absence of female NSG mice. Further, observations may be strengthened by using a larger data set. In addition to the stated limitations, it is important to mention ways that our data could be more comprehensive. Since the flow cytometry data provided double positive percentages for both c-peptide and NKX6.1, our data would be

strengthened by including histochemical staining for NKX6.1 in addition to the stained c-peptide. Nonetheless, our preliminary data suggests that it may be valuable to further research the differences between SC-βs of different positivity rates as well as the maturational capacity of these cells to enhance the patient experience in the clinic.

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