Targeting Apoptosis and Necroptosis Inhibition to Improve Islet Graft Function

by

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Abstract

Islet cell transplantation is an effective method to treat type 1 diabetes mellitus, however, significant barriers currently exist preventing its implementation as the gold standard therapy. Of note, cell death in the acute post-transplant period accounts for loss of up to 80% of newly transplanted islets. Cell death can be mediated in multiple different ways, and the two primary forms of cell death are apoptosis and necrosis. Significant efforts have been applied in preventing apoptosis following islet transplantation, however, concurrent inhibition of both pathways simultaneously is a relatively new approach. In this thesis, we investigated the effect of apoptosis and regulated necrosis inhibition on β-cell survival and function, both *in vivo* and *in vitro.* MIN6 cells, derived from an immortalized mouse insulinoma, were cultured with and without apoptosis (ZVAD-FMK) and regulated necrosis inhibitors (Necrostatin 1, Necrostatin 1s) for 24-72 hours, and under basal and stressed conditions. Cell vitality, glucose stimulated insulin secretion, and oxygen consumption rates were measured and analyzed. This revealed statistically significant differences between treated and untreated cells, suggesting the therapeutic potential of treating islets with apoptosis and regulated necrosis inhibitors prior to islet transplantation.

Next, human islets were treated for 24h with apoptosis and regulated necrosis inhibitors, and marginal mass transplantation of these islets under the kidney capsule of diabetic mice was performed. Mice were followed for 60 days, and improved reversal of diabetes and maintenance of glycemic control was observed in mice treated with the inhibitors in combination and alone. This indicates that pre-treatment of islets with apoptosis and regulated necrosis inhibitors prior to transplantation may reduce cell death following transplantation, and potentially improve islet engraftment. This is of great clinical relevance, as targeting regulated cell death effectively may

in the future lead to improved single donor success rates, reduced islet mass required in each transplant, and long-term insulin independence following the procedure.

Preface

This thesis is an original work by Saloni Aggarwal. Parts of Chapter 1 of this thesis have been published as S. Aggarwal, A.R Pepper, G.S Korbutt, "Clinical translation of porcine islets for treating type 1 diabetes" in the journal Current Opinion in Endocrine and Metabolic Research (**Appendix A-1**), and as S. Aggarwal, A.R Pepper, N. Al Jahdhami, "Augmenting engraftment of beta cell replacement therapies for T1DM" in the Journal of Immunology and Regenerative Medicine (**Appendix A-2**). As part of the laboratory responsibilities and learning opportunities associated with this project, Saloni Aggarwal was involved in assisting Haopeng Frank Lin in an OCR assay, and this work is reflected in the published paper, "Redox sensing by SENP1 augments insulin secretion early after high-fat feeding in mice" by H. Lin, K. Suzuki, N. Smith, X. Li, L. Nalbach, S. Fuentes, A.F. Spigelman, X. Dai, A. Bautista, M. Ferdaoussi, S. Aggarwal, A.R Pepper, L.P. Roma, E. Ampofo, W. Li, P.E. MacDonald (**Appendix A-3**).

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List of Symbols

α – Alpha

β – Beta

δ – Delta

ε – Epsilon

γ – Gamma

μ – Mu

Glossary of Terms

A20 - TNFAIP3; TNF-α-induced protein 3

AAT - Alpha-1-antitrypsin

ARNT - Aryl hydrocarbon receptor nuclear translocator

Atk - Protein kinase B/PKB

ANA - Interleukin-1 receptor antagonist

ATP - Adenosine triphosphate

AUC - Area under curve

Bax - Bcl-2 Associated X-protein

BCL-2 - B-cell lymphoma 2

BMI - Body mass index

C5aIP - Complement 5a inhibitory peptide

cFLIP - Caspase-8 and FADD-like apoptosis regulator; cellular FLICE - like inhibitory protein

CIT - Clinical Islet Transplantation

CITR - Collaborative islet transplant registry

CNI - Calcineurin inhibitor

CO_2 - Carbon dioxide

DAMP - Damage-associated molecular patterns

DCD - Donation after cardiac death

DM - Diabetes Mellitus

DMEM - Dulbecco's modified Eagle's medium

DNA - Deoxyribonucleic acid

DSA - Donor-specific anti- HLA antibodies

dsDNA - double stranded DNA

ECM - Extracellular matrix molecules

E3 - Ubiquitin ligase

ER - Endoplasmic reticulum

ERK - Extracellular signal-regulated kinases

ETA - Entanercept

FADD - Fas-associated protein with death domain

GPX1 - Selenium-dependent glutathione peroxidase 1

GLP-1 - Glucagon-like peptide 1

GLUT 2 - Glucose transporter 2

GSIS - Glucose Stimulated Insulin Secretion

HbA1c - Hemoglobin A1c

HIF1-α - Hypoxia-inducible factor-1-α

HIF1-β - Hypoxia-inducible factor-1-β

HLA - Human Leukocyte Antigen

HO-1 - Heme-oxygenase-1

HRE - Hypoxic response element

HMGB1 - High mobility group box 1 protein

hPSCs - human pluripotent stem cells

IAH - Impaired awareness of hypoglycemia

IBMIR - Instant blood mediated inflammatory reaction

ICU - Intensive care unit

IDO - Indoleamine 2,3-dioxygnase

IL-1β - Interleukin- 1-β

IL2 - Interleukin-2

IL-6 - Interleukin-6

IPGTT - Intraperitoneal glucose tolerance test

iPS - induced pluripotent stem cells

IRE1 - Inositol Requiring 1

IRI - Ischemia reperfusion injury

LDHA - Lactate de-hydrogenase A

LMW-DS - Low molecular-weight dextran sulfate

LPS - Lipopolysaccharide

MHC1 - Major Histocompatibility Class 1

MIN - Mouse Insulinoma

MLKL - Mixed lineage kinase domain-like protein

mRNA - Messenger RNA

MSC - Mesenchymal stem cells

Nec - Necrostatin

NF-kB - nuclear factor kappa B

NLRP3 - NLR family pyrin domain containing 3

PAK - Pancreas after kidney transplant

pO2 - Oxygen partial pressure

P-PASS - Pre-procurement pancreas suitability score

PTA - Pancreas transplant alone

RIPK1 - Receptor interacting serine/threonine-protein kinase 1

RIPK3 - Receptor interacting serine/threonine-protein kinase 3

ROS - Reactive oxygen species

RNA - Ribonucleic acid

SHE - Severe hypogylcemic events

siRNA - Short interfering RNA

SOD1 – Cu, Zn-superoxide dismutase 1

SPK - Simultaneous kidney and pancreas transplants

STZ - Streptozotocin

T1DM - Type 1 Diabetes Mellitus

T2DM - Type 2 Diabetes Mellitus

TLR3 - Toll-like receptor 3

TLR4 - Toll-like receptor 4

TNF - Tumour necrosis factor

TNFR1 - Tumour necrosis factor receptor 1

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

VHL - von Hippel-Lindau

ZVAD; Z-VAD-FMK - benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone

CHAPTER 1: AN INTRODUCTION TO B-CELL REPLACEMENT THERAPIES AND ASSOCIATED CHALLENGES

1.1 Diabetes Mellitus

Endocrine function takes place within the Islets of Langerhans, which comprise 2% of the pancreas by mass. These islets release various hormones into the bloodstream to regulate blood glucose levels. Humans have approximately one million islets distributed throughout the pancreas.[1] The islets are composed of five different cell types. Insulin-producing β-cells are the first detectable differentiated cell present in the pancreas,[2] and make up approximately 60% of the human islet. Insulin regulates the metabolism of carbohydrates, fats, and protein by promoting glucose absorption from the bloodstream and into the liver, fat, and skeletal muscle cells, where it is primarily converted to glycogen. β-cells also produce amylin, which slows gastric emptying to reduce post-prandial spikes. Glucagon-producing α-cells make up 30% of the islets. The remainder of the islets are composed of δ-cells which produce somatostatin to inhibit insulin and glucagon, γ-cells which produce pancreatic polypeptide to regulate pancreatic secretion and is thought to have some relation to hunger cues, and ε-cells which produce ghrelin to increase gastric motility and stimulate gastric acid secretion.[3] These cells all work together to maintain glucose homeostasis in the body and communicate via paracrine signalling and through gap junctions.[3]

Under normal physiological conditions, the cells of the pancreas are able to detect changes in blood glucose levels and secrete various hormones as appropriate. Glucose enters the β-cells through GLUT 2 transporters and is metabolized, producing ATP, and increasing the ATP:ADP ratio of the cell. This closes the K-ATP channels in the cell, and opens the voltage gated calcium ion channels, inducing insulin release.[4] Specifically, fusion of intracellular secretory vesicles containing insulin granules with the plasma membrane occurs, and subsequent

exocytosis of these granules results in insulin secretion.[4] β-cells are also responsive to the presence of protein and fat, albeit at a much lower degree than they are to glucose.

However, in cases of abnormal signal-receptor connections at any part of the process, diabetes mellitus (DM) can develop. DM encompasses a group of metabolic diseases, including but not limited to type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes, maturity onset diabetes of the young (MODY), and pancreatogenic diabetes. During the past decades, the prevalence of diabetes mellitus has increased significantly according to the World Health Organization. As of 2019, 463 million people are suffering from the disease, and a continuous rise of 10.9% is expected through to 2045.[5] Given the increasing prevalence of this disease which affects both the Canadian and global populations, there is a pressing need to better understand the underlying physiology behind DM development, and also develop comprehensive therapeutic strategies in order to manage both DM and its associated complications.

1.2 Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM), once known as juvenile-onset diabetes, compromises approximately 10% of people with diabetes.[6] T1DM is a chronic autoimmune disorder characterized by selective and progressive destruction of insulin producing β-cells, within the pancreatic islets of Langerhans, and results in β-cell depletion, overt hyperglycemia and insulin dependence. Characteristics of T1DM diagnosis include hyperglycemia, low, or undetectable C-peptide levels, increased hemoglobin A1c (HbA1c), and positive autoantibody markers accompanied by polyuria, polydipsia, polyphagia, and weight loss.[7] T1DM cannot be prevented through alterations in lifestyle.

Predisposition to T1DM has been linked to several genetic and environmental factors, and infectious triggers have been implicated in the autoimmune attack on the β-cells.[8, 9] Studies have shown that certain genes within the major histocompatibility complex (MHC) such as human leukocyte antigen (HLA)-DQ locus, located on the short arm of chromosome 6, contribute to one half of the susceptibility to the disease.[10] Additionally, studies in prospective cohorts have suggested that the appearance of islet antibodies that precede high glucose levels in T1DM is due to enterovirus infections such as Coxsackie virus B, and other viruses such as mumps, cytomegalovirus, and congenital Rubella syndrome,[11] which in turn induce a higher cellular immune response.[12] It is hypothesized that these viruses function through a process of molecular mimicry and bystander T-cell activation causing inflammation. Coxsackie virus B in particular shares sequence homology with the islet autoantibody glutamic acid decarboxylase.[13] Environmental triggers lead to an increased expression of major histocompatibility complex class 1 (MHC1) on resident β-cells, leading to their destruction by autoreactive T cells. Additionally, activation of pro-inflammatory triggers, such as CD4 T cells,

generates the production of β-cell specific autoantibodies, and induces cytotoxic CD8 T cell migration to destroy β-cells within the islet.[14-16]

Impaired awareness of hypoglycemia (IAH) occurs when blood glucose levels drop steeply without a patient recognizing the symptoms. Severe hypoglycemia often presents with physical symptoms including but not limited to heart palpitations, shivering, shaking, sweating and cognitive dysfunction. Recurrent episodes of hypoglycemia impair the regulatory responses to hypoglycemic episodes, leading to more frequent and severe instances of hypoglycemia (severe hypogylcemic events, SHE), and an overall reduction in the body's ability to recognize these events.[17] Without rapid and effective intervention, hypoglycemia can quickly lead to an individual experiencing seizures or loss of consciousness, progressing to coma or death.[18] It is estimated that 4–10% of people with T1DM die of hypoglycemia.[19] Additionally, a patient may experience 'unstable' or 'brittle' diabetes, characterized by unpredictable and frequent swings from high to low blood glucose levels, which makes it difficult to maintain any semblance of stable glucose levels. Both situations can be fatal, and as such, require urgent resolution. Aside from the requisite of systemic immunosuppression, additional barriers need to be vanquished before β-cell replacement therapies can be offered to all patients with T1DM. These barriers include poor β-cell engraftment rates, variability in islet isolation and transplant outcomes, inability to achieve routine insulin independence in comparison to the pancreas, heterogenicity in islet functional potency, and suboptimal transplant microenvironments.

1.3 Insulin Replacement Therapy

Individuals with T1DM manage their diabetes through daily insulin injections,[20] however, it is difficult to maintain truly optimal blood glucose levels. Alternative therapies such as continuous glucose monitoring systems, and insulin and bi-hormonal pumps can aid in reducing the glycaemic fluctuation. Randomized clinical trials and cohort studies have shown that continuous glucose monitoring can reduce the risk of hypoglycemia.[21] Additionally, a double-blind randomized cross over trial showed that achieving near normal glucose concentration is possible by delivering new analogue insulins such as fast or standard insulin aspart by a fully closed loop method, which was independent of hypoglycemia.[22] Nonetheless, tighter glycaemic control has been clearly associated with an increased frequency of unintentional hypoglycaemia, which may lead to serious complications, cognitive impairment, coma, and death.[23] Alternatively, hypoglycaemia risks can be reduced through less intensive exogenous insulin therapy, leading to sub-optimal or poor control of glucose levels, and this in turn increases the risk of diabetes related vascular complications including retinopathy, neuropathy, nephropathy, and ultimately end-stage organ disease.[24] Thus, despite advancements in exogenous insulin therapy, optimal medical management is challenging as all modalities imprecisely recapitulate the tight glucose regulation achieved by the pancreas.

Dynamic glucose sensing and matched insulin response can only be restored via β-cell replacement therapy, which is currently achieved with either whole pancreas transplantation or islets of Langerhans transplantation.[25] Pancreas transplantation involves major abdominal surgery with significant peri-operative morbidity and mortality rates, leading to surgery risks, and the requirement of life-long immunosuppressive therapy.[25, 26] Islet transplantation provides a minimally invasive β-cell replacement therapy for T1DM, with incremental

improvements in patient outcomes due to advancements in islet isolation techniques and immunotherapy.[27] Islet transplantation as a therapeutic replacement modality has been shown to be particularly effective, yet restricted, in patients who suffer recurrent severe hypoglycemia,[28] as the post-transplant metabolic benefits outweigh the potential risks associated with life-long immunosuppression including organ dysfunction, opportunistic infections and malignancy. Approximately 5-10% of patients with T1DM experience recurrent SHEs, with the Scotland DARTS study reporting a percentage of 7.1%.[18] One study that followed 12 islet transplant patients observed maintenance of glycemic control over the full 18 months that follow-up was conducted.[29] A multi-center phase 3 study observed that 88% of patients were free of all SHEs after one year following an islet transplant, and 71% of patients were free of all SHEs after two years.[30]

1.3.1 Identification of Pancreatic Function

The Islets of Langerhans were first identified within the pancreas of a rabbit, by Paul Langerhans in 1869, a medical student in Germany at the time, who described their structure but was unable to determine their function.[31] In 1893, French doctor Gustave-Edouard Laguesse discovered that the islet cells produce a substance which aids in digestion, coining the term "endocrine secretion."[32] In addition, experiments performed by Leonid Ssobolew in 1901 indicated that ligation of the pancreatic ducts in rabbits, cats, and dogs led to destruction of the acinar cells but preservation of the islet cells, with no increase of glucose in the urine. This suggested that the islet cells were key in treating diabetes.[33]

It wasn't until 1920 that Frederick Banting, working under Dr. John Macleod, set out to investigate the nature of the pancreatic secretions. Taking undergraduate student Charles Best as

his assistant, the two worked to ligate the pancreatic ducts of a dog, extract the islets following acinar cell degeneration, then inject these islets into a diabetic dog. Successful injection led to near-immediate reduction in blood glucose levels, along with reduction of ketone bodies and glucose in the urine. Furthermore, administration of an excess of the extract lead to hypoglycemia.[34] Following these early successes, the team was joined by Dr. James Collip, a biochemist who worked to concentrate and purify the fresh pancreatic extracts in preparation for human trials.[33] On January 11th, 1922, the first patient was treated with 15mL of this extract, however, due to its impurities, it was ineffective, and in fact, an abscess developed at the site of injection.

Following further modification and purification by Collip, another patient was injected with the extract on January 23rd. This was the first successful clinical treatment of type 1 diabetes. Banting and MacLeod were awarded a joint Nobel prize for their work in April of 1923, and the two shared the monetary award with Best and Collip. In 1972, the Nobel Prize Committee acknowledged that a mistake had been made in excluding Best from the Prize.[33]

1.3.2 Whole Pancreas Transplantation

By the early 1900s, successful attempts at skin, corneal, and thyroid transplants lent validity to the idea that a diseased body part in one individual could be replaced with a healthy part taken from another individual. Larger and more complex organ transplants were made possible by the foundations laid by Dr. Alexis Carrel, who made significant advances in the field of vascular anastomosis, allowing for the repair of torn or divided blood vessels, and revolutionizing vascular surgery in the process. A gifted surgeon, he went on to conduct transplant-related experiments on dogs and cats, identifying the issue of graft rejection in the

process. Further medical progress was stalled at this juncture given that explanations for failures in homografts could not be found, and both xenotransplantation and allotransplantation attempts over the next few decades were largely unsuccessful. Pancreas transplantation in an animal model was first attempted in 1913 – an allograft was placed in the neck of a dog that had previously undergone a pancreatectomy. This graft failed immediately without the reversal of diabetes, and repeated experimentation was unsuccessful until 1927, when transient reversal of diabetes using the same method was accomplished by Gayet and Guillaumie, a French research group. They concluded that previous experimentation in this manner was conducted with poor precision.

The first recorded attempt at major organ transplantation from a human donor to human recipient was performed in 1936, by Dr. Yu Yu Voronoy, a Ukranian surgeon who transplanted a kidney from a deceased donor to an individual suffering from acute renal failure. The kidney failed to produce any urine, turned black shortly after transplant, and the patient died within 48 hours of the surgery. Three factors jeopardized the success of the transplant – the donor and recipient were of different blood types, the recipient was still suffering from residual mercury poisoning, and the kidney was retrieved from the donor 6 hours after the donor’s death. Voronay performed four other unsuccessful human homografts between 1933 and 1949, and teams working separately in Paris, Denmark, and the United States faced similar results within the same time period. Although it was evident that kidney rejection was causing failure, lack of kidney dialysis machines or other renal-failure treatments justified the continual performance of transplants despite poor outcomes.

Almost coincidentally, in the mid-1940s, a zoologist, Peter Medawar, was sent to a Glasgow hospital to assist in the treatment of burned pilots. He observed repeated failures of

homografts, and noted that second grafts from the same donor failed more quickly than the first. Although Medawar was not solely responsible for this finding, it is often attributed to him. This, along with his further research in the characterization of the timing, morphology, and nature of rejection, led to a Nobel Prize in 1953, for describing the process of acquired tolerance.[35]

With developing knowledge of surgical and medical techniques, as well as with the use of immune suppressing medication, the first successful transplant between two non-twin individuals was performed in 1960. The 1960s also saw the first use of immunosuppressive agents. Initial strategies used to combat rejection involved total body irradiation and adrenal cortical steroids, but they had low success, or had high toxicity levels. The introduction of the use of Cyclosporin A in 1979 was a large step forward in the advancement of transplantation, especially when used in conjunction with azathioprine and other steroids.

Developments and improvements in surgical procedures have been instrumental in ensuring positive outcomes. A pancreas transplant was first attempted at the University of Manitoba in December of 1966 by Drs. Kelly and Lillehei and resulted in the achievement of insulin independence for six days. Following laboratory research, significant changes in surgical technique were made during the second transplant attempt. Graft attrition occurred at a slower rate, but the patient died of sepsis 4.5 months later. Of thirteen total attempts by this team, only one graft remained functional for over a year.

Pancreas transplants experience higher post-op complications in comparison to liver and kidney transplants. Whole organ transplants also come at the cost of significant complications and surgical risks, including thrombosis, infection, and rejection.[36] By the beginning of the 1980s, 105 pancreas transplants had been performed in total, and success rates of simultaneous

kidney and pancreas transplants (SPK) vs pancreas transplants alone (PTA) indicated decreasing mortality rates with SPK transplants over time but better success with PTA transplants overall.

Continued tracking of these rates, as well as the inclusion of pancreas after kidney transplants (PAK) have led to the observance of differential rates of success. It is currently recommended that whole-pancreas transplants be performed in patients with end-stage renal disease who are also requiring a kidney transplant. When performed early, kidney transplant leads to markedly higher survival rates 5 years post-transplant.[37] Dual transplants also result in better success rates than pancreas transplants alone.[38] Autotransplantation of islet cells alone is a commonly performed procedure for patients who have had pancreatectomies to treat pancreatitis, but as T1DM is an autoimmune disease which attacks the body's own cells, this is not an option for T1DM patients.

1.3.3 Islet Transplantation

Initial attempts of islet transplantation by Watson-Williams were reported in early 1893, with the insertion of minced sheep pancreata into the thigh of a young patient with diabetic ketoacidosis. Despite improvement in glycosuria after 24h, the procedure failed due to the lack of systemic immunosuppression, and the development of ischemia at the transplant site.[39] Nearly a century later, the next disruptive milestone was reached by Paul E. Lacy in 1972, who achieved insulin independence in rats with chemically induced diabetes, by infusing 400–600 islets through the intraportal vein.[40] In 1980, clinical translation of islet transplantation was accomplished through successful autografts of 10 patients via intraportal islet transplantation, with 3 patients achieving insulin independence for 1, 9, and 38 months.[41] Despite this tantalizing achievement, extensive clinical application of islet transplantation was plagued by the

inconsistent isolation of high yields of transplant-grade human pancreatic islets. From the 1980s onward, improvements in immunosuppression made whole organ transplantation clinically achievable. In 1989, Ricordi and colleagues were able to develop a semi-automated continuous digestion-filtration human pancreas processing method, leading to routine high yield and purity of human islets.[42] This enabled a collaborative clinical trial in the early 1990s, which reported the first successful transient period of insulin independence after an allotransplant.[43]

Despite these efforts to achieve insulin independence, less than 8% of the subsequent 267 islet transplant attempts were successful for more than one year, until the breakthrough of the "Edmonton protocol" in 2000.[44, 45] The Edmonton group reported for the first time, 100% insulin independence at 1-year post transplantation in a series of 7 patients, by adoption of a steroid-free immunosuppressant therapy and by transplanting a large dose of human islets. However, the loss of complete insulin independence was observed between years 3–5, with only 10% of patients remaining insulin free after 5 years, despite 80% maintaining C-peptide production with normalized HbA1C, and experiencing protection from severe hypoglycemia.[45, 46] Nevertheless, the success of the Edmonton protocol led to its adoption in many centers internationally, and aided in establishing the Collaborative Islet Transplant Registry (CITR). Furthermore, it was later reported that durable long-term function is achievable, as approximately 50% of patients remained insulin independent with prolonged C-peptide production at 5 years post-transplant when TNF anti-inflammatory treatment was administered in conjunction with T-cell depletion.[47]

Most recently, a follow up Phase 3 clinical trial transplanted purified human pancreatic islets into forty-eight adults with T1DM with IAH and SHEs led to restoration of near-normal glycemic control, restoration of hypoglycemia awareness, and protection from SHEs in subjects

with intractable IAH. These successful outcomes were achieved in 87.5% and 71% of participants at Year 1 and Year 2 respectively, and median HbA1c levels of 5.6% were achieved.[30] This pivotal study and other similar ones have clearly demonstrated that islet transplantation provides superior glycemic control with reduced hypoglycemic events, compared with standard insulin therapy.[48] The 2000 study which established the Edmonton Protocol additionally observed that the mean number of islets transplanted in patients who achieved insulin independence was 11,547±1604 IEQ per kilogram of recipient's body mass,[45] which was subsequently approximated to at least 10,000 IEQ/kg in further transplant trials, both in Edmonton and Internationally. Although the aim of the Edmonton trial and these other preliminary trials was to restore independence from exogenous insulin, these goals have been modified in recent years.[49] The primary endpoint of an islet transplant is now considered to be the absence of severe hypoglycemic episodes with return to maintenance of glycemic control (HbA1C < 7%).[50] Moreover, clinical trials show that islet transplantation results in less progression of diabetic retinopathy and nephropathy, in comparison with intensive medical therapy.[51] However, several obstacles still remain, restricting this life-altering therapy to a narrow spectrum of patients with T1DM.

1.3.4 Sources of Islets

Shortages of human islet donors, low rates of single donor transplant success, and early islet destruction through the instant blood mediated inflammatory reaction (IBMIR)[52], leads to the necessity of considering other islet sources. Human stem cell-derived islet clusters generated from induced pluripotent stem cells (iPS) or human pluripotent stem cells (hPSCs) represent a virtually unlimited source to generate islets for diabetes therapy.[53] Utilization of these cells

triggers fewer ethical concerns and the potential for autologous transplantation. However, these cells are limited by the mutations that are introduced during reprogramming and expansion, often do not completely recapitulate native islet function, and the production of cells that do not trigger the autoimmune response has proven to be difficult and time consuming.[54] Advancements in protocols by Vertex has led to a Phase I/II multi-center clinical trial (NCT04786262), using VX-880, which are allogenic stem cell-derived, insulin-producing islets.

SC-islets have also been studied in transplant models using microencapsulated and microencapsulated forms.[55, 56] In microencapsulation, single, or small groups of islets are typically encased in alginate. These encapsulated structures maximize the surface area to volume ratio to improve oxygen and nutrient diffusion,[57] and permit β-cell maturation and differentiation.[58] Implantation of these structures is typically both safe and minimally invasive, however, removal of the grafted cells if necessary, can be difficult.[59] Alginate microencapsulation of hPSCs in vitro improves the percentage of insulin-expressing cells co-expressing key β-cell markers, and islets encapsulated at different phases of development will produce different enzymes and differentiation markers.[60]

Comparatively, macroencapsulation involves encasing a large number of islets within a single semi-permeable chamber. Within the device, islets can exist as clusters, permitting communication between the cells as it occurs in a healthy pancreas. The semi-permeable membrane permits for the growth of blood vessels towards and within the device, which allow for nutrient supply and the exchange of insulin and glucose.[61] However, as the surface area to volume ratio is low, diffusion is slow, leading to an insufficient supply of nutrients.[62] Islet insulin production halts if cells must compete for oxygen,[63] and so islet mass cannot exceed 5-

10% of device volume. Lack of oxygenation also creates risk for cell death and the development of central necrosis. Extravascular implants can be implanted intraperitoneally or subcutaneously, without direct vascular access. The implantation procedure is minimally invasive, carries with it less complications, and the device can be removed or replaced if necessary.

Early clinical transplant of extravascular macrocapsules led to failure due to fibroblast overgrowth within and around the chamber,[64] or grafted cell death due to inadequate oxygenation.[59] More recently, commercial devices have led to promising clinical results. In 2014, Viacyte launched a phase I/II trial (NCT02239354), involving the implantation of human embryonic stem cells (PEC-01 cells) within an Encaptra device, which involves a macroencapsulation device comprising of a thin impermeable inner membrane and permeable outer support matrix to protect implanted cells without the use of immunosuppressive medication. Following engraftment and vasculogenesis, PEC-01 cells were observed to mature into insulin producing β-cells, without any adverse effects observed at the two-year mark, however, glycemic control was not demonstrated. These encouraging results led to the initiation of a second trial in 2017 (NCT03163511) with the goal of improving cell survival, but involving the provision of systemic immunosuppression.[65] This study is still currently underway, with an estimated primary completion date of November 2023.

Other devices are also currently being trialed. The Beta-O2 device comprises of two modules, an islet-containing capsule which is surrounded by a three-layer membrane which allows free diffusion of oxygen, glucose, and insulin, and an oxygen port which allows manual operation to allow for infusion of gas into the capsule. Diabetic rats transplanted with this device experienced normoglycemia until the device was explanted at day 240,[66] however transplantation into a T1D patient did not produce similar results, although further research is

ongoing (NCT02064309).[67] These first in human trials have the potential to radically transform the landscape of cell-based therapies for the treatment of T1DM.

Alternatively, there is a strong rationale to pursue the use of porcine donors for clinical islet xenotransplantation,[53] including: 1) the unlimited availability of porcine islets, increasing access to islet transplants and eliminating waiting time; 2) the reproducibility and quality of preparing porcine islets, predictably high and not compromised by co-morbidity, brain death and ischemia related to human islets; 3) porcine insulin has been used to treat human diabetes for more than 90 years; 4) porcine islets respond to glucose in the same physiological range as do human islets; 5) new techniques allow genetic manipulation and cloning of pigs, if it proves necessary or advantageous to do so; and 6) porcine islets are a potential therapy for highly allosensitized patients[68]. Furthermore, neonatal porcine islets are not susceptible to amyloid deposition, which results in apoptosis. Amyloid aggregation occurs rapidly in newly transplanted human islets and contributes to graft loss.[69] Thus, the risk-benefit ratio of porcine islet grafts makes them a major therapeutic option to the currently used human islet grafts[70].

1.4 Challenges to Islet Transplantation

Despite marked improvement in clinical islet transplantation, durable insulin independence may not be routinely achieved due to the reduction in β-cell mass attributed to several factors, including acute islet graft loss due to the activation of the instant blood mediated inflammatory reaction (IBMIR) following intrahepatic infusion. A major impediment to islet transplantation is that anti-rejection immunotherapies are required lifelong to prevent rejection-mediated cell loss, and consequently, transplantation has not been universally embraced as a therapy for broad application.[71] Beyond immunosuppression challenges, other major hurdles confronting islet transplantation are associated with islet isolation from the donor pancreas, typically resulting in the requirement of multiple pancreata per islet recipient to achieve insulin independence. Isolation requires multiple steps, beginning with preservation of the procured pancreas, followed by intraductal distention with collagenase and digestion in a Ricordi chamber, and finally purification of the isolated islets from the exocrine tissue via a density gradient centrifugation. Prior to transplantation, the isolated islets must undergo quality control tests, and show adequate purity, dose (>10,000 IEQ/kg of recipient body weight), and a settled tissue volume <7 cc.[6] Accordingly, the process of islet isolation and transplantation is hindered by many challenges which could eventually lead to graft failure. Furthermore, many questions remain to be addressed in terms of the optimal transplantation site, due to low oxygen tension in the intraportal vein. Relative hypoxia is associated with a loss of β-cell mass and vasculature, and eventually leads to necrotic and apoptotic β-cell death. Prior to islet transplantation, the isolation process subjects islets to deleterious stressors, where their extracellular and vascular niche is disrupted, inevitably leading to reduced β-cell mass engraftment. As such, methods to

improve the functional quality and success rates of islet preparations may indeed translate to more favourable transplant outcomes.

1.4.1 Islet Vasculature

Although islets make up 1–2% of the pancreas by mass, they receive approximately 10% of the pancreatic blood supply, as islets have high oxygen and nutrient requirements. Islet vasculature plays an important role in the ability of islets to respond to blood glucose changes and secrete insulin. Critical to their ability to regulate glycemia, native islets reside within a complex network of arterioles and capillaries, ensuring that each islet is never more than one cell away from arterial blood. Blood vessels of greater density within pancreatic islets lined with fenestrated endothelial cells are notable in comparison to blood vessels surrounding the exocrine tissue; thereby providing islets with greater partial oxygen pressure in comparison with other organs and acinar tissue.[72, 73] Pancreatic differentiation and morphogenesis are instructed by the development of reciprocal endothelial-endocrine cell signaling and the formation of functional blood vessels during embryonic development.[72, 74, 75] Islet formation is coordinated with the development of islet vasculature, with assembly into mature islets requiring blood flow to the endocrine cells.[73] Enhancement of islet survival and the outcomes of islet transplantation may be achieved through the development of modalities directed towards enhancing the revascularization of transplanted islets.

During islet isolation the connection between islet vasculature and systemic circulation is disrupted, therefore acute stimulation of angio- genesis and possibly vasculogenesis will be required to re-establish blood flow to the core of transplanted islets. However, after post-transplant revascularization, islets are predisposed to reduced vascularization, and have a lower oxygen tension compared to endogenous islets in the pancreas.[76, 77] In the immediate post-

transplant period, islet metabolic exchange is limited to passive diffusion. Dynamic revascularization of islets following transplant, which re-establishes the endogenous network, culminates in mature vessel formation approximately two weeks post-infusion through angiogenesis and vasculogenesis. Endothelial cells from the transplant recipient's own bone marrow form vasculature together with intra-islet endothelial cells from the graft to create new chimeric capillaries. Even once blood flow is re-established, transplanted islets still experience chronically low vascular density and blood perfusion, and consequently, low oxygen tension (5 mmHg) as compared with native islets within the pancreas (40 mmHg). This phenomenon is consistent despite the location of transplant – implantation in the kidney cortex, liver, and spleen experienced similar islet graft blood perfusion, despite the three organs receiving different levels of endogenous perfusion.[76] Ischemia and inadequate blood supply lead to cell death, which impairs islets survival and function post-transplantation. Strategies targeted toward neo-vascularization stimulation and inflammation dampening are promising means to support islet cells survival in the post-transplant period.

1.4.2 Extracellular Matrix

During the isolation procedure, enzymatic digestion of pancreatic tissue via collagenase, and mechanical digestion, necessarily breaks the cell-cell adhesion between exocrine and endocrine cells, but also collaterally damages extracellular matrix molecules (ECM), affecting islet function.[78, 79] Extracellular matrix molecules provide mechanical and physiological support to islets, which has the potential to promote islet cell survival and function. Collagen provides structural stiffness and cohesiveness to the tissue, and is found in the peripheral ECM of mature islets, within the islet-acinar interface, and in islet basement membranes.[80] Fibronectin

facilitates cell adhesion, contractility, and cytoskeleton remodelling, and regulates islet processes through interaction with integrin receptors expressed by islets. Laminin aids in maintaining organ structure, while fibronectin and laminin aid in the differentiation of mesenchymal stem cells (MSCs) into insulin producing cells through activation of the Atk and ERK pathways.[81] The ECM can also serve as a reservoir for immunomodulating signals, such as cytokines or growth factors, which protect against β-cell death.[82] Detachment of anchored cells from the surrounding ECM results in anoikis, a programmed form of cell death, which contributes to loss of functional islet mass. Anoikis and similar cell death processes are accompanied by the release of damage-associated molecular patterns (DAMPs), which are immunostimulatory and when secreted or exposed on the cell surface, release signals which recruit immune cells. Stimulation of this inflammatory response is partially associated with graft failure.[83]

Attempts at improving revascularization *in vivo* are challenging, as mechanisms of damage and subsequent repair are not well understood thus far. It is known that revascularization involves basement membrane and extracellular matrix remodelling, as well as the involvement of support cells such as pericytes. Co-culture of islets with ECM molecules has been shown to improve the graft survival and function,[84] as the ECM molecules mimic the biochemical composition, fibrillar structure, and viscoelastic properties of the ECM in the target organ.[85] Encapsulation of MIN6 cells in gel environments containing Collagen I, Collagen IV, fibronectin, and laminin resulted in increased cell survival and decreased apoptosis. Collagen IV and laminin gel encapsulation induced an increase in glucose stimulated insulin secretion (GSIS) when monitored over 10 days.[84] However, at high concentrations, Collagen IV has been observed to impair GSIS, decreasing insulin production in β-cells.[80]

Decellularized pancreata can provide a scaffold on which to support transplanted islets within the body. Decellularization of tissue provides the advantage of reducing immunogenicity, while leaving behind ECM molecules which would support the islets. *In vitro* data has indicated that islet function and survival is maintained when cultured on decellularized pancreata.[86, 87]Furthermore, infusion of mouse islets into decellularized lung tissue, and transplantation into the peritoneal cavity of diabetic mice resulted in enhanced restoration of euglycemia.[88] Consequently, co-culture with ECM molecules, or co-transplant with decellularized scaffolds may improve islet survival and function *in vivo*. At the same time, certain ECM molecules, such as matrix metalloproteinases, cleave and degrade ECM proteins, along with other cytokines, chemokines, growth factors, and cell adhesion molecules, leading to impaired cellular growth and differentiation.[80] Indeed, strategies directed towards restoring native ECM in islets are promising but must be finely tuned so as not to exacerbate islets susceptibility to immune mediated destruction.

1.4.3 Donor considerations

Due to human islet donor heterogenicity, proper patient selection may be critical for achieving optimal post-transplant outcomes. HLA class I and II molecules regulate the immune system and assist the body in differentiating self vs non-self. The emergence of donor-specific anti- HLA antibodies (DSA) are recognized as one of the causes of solid organ transplant failure,[89, 90] including whole-pancreas transplant failure.[91, 92] Several reports have also indicated the development of DSA following islet transplantation.[93, 94], [95] Despite T-cell depletion therapy, research indicating the correlation between the appearance of DSA and islet graft failure is limited, [96, 97] It has also been suggested that islet grafting is an HLA-sensitizing event for recipients, [98] particularly in patients that discontinue immunosuppressive

drugs.[95] Sensitization occurs due to the absence of HLA matching at the time of transplantation and the subsequent development of non-self-anti-HLA antibodies, exacerbated by the use of multiple donors per transplant to obtain sufficient mass for transplantation.[99] Post-transplant systemic immunosuppression is insufficient to improve single-donor success rates, or to prevent the immune response generated by the HLA mismatch.[100] It has been observed that patients who experience a statistically significant increase in DSA following islet transplant are >5× more likely to develop graft failure, as compared with patients that did not experience DSA increase.[101] This is problematic as HLA sensitization potentially precludes future transplant procedures, including islet, pancreas, kidney, or other organs if needed.[102, 103] Progressive graft attrition is observed in allografts, such that only 50% of patients remain insulin-independent by five years post-transplant, necessitating multiple transplant procedures. T1DM patients are also at risk for the development of diabetic nephropathy and may require a future kidney transplant. Preventing graft attrition and achieving single-donor insulin independence could mitigate these harms.

Furthermore, individual donor characteristics influence transplant outcomes. Although differences in islet yield were not observed, engraftment function parameters such as C-peptide/glucose ratio, secretory units of islets in transplantation index, β scores, and insulin independence rates at 1, 6, and 12 months post-transplant, were all improved when donors were under the age of 45.[104] However, digestion of pancreata of young individuals carries additional technical difficulty, due to its fibrous nature.[105] Pancreata obtained from higher BMI-individuals allow for higher yields of islets, potentially due to increased islet mass or larger islet size.[106] At the same time, care must be taken to avoid donors with type 2 diabetes mellitus (T2DM), as β-cells from T2DM patients function poorly following transplant.[107] The

Euro- transplant Pancreas Advisory committee established a pre-procurement pancreas suitability score (P-PASS), taking into consideration age, BMI, duration of ICU stay, cardiac arrest, serum sodium, amylase, lipase, and catecholamine dose, and recommended that only donors with a P-PASS of less than 17 be considered for donation.[108, 109] In practice this has variable results – in Poland, retrospective analysis of P-PASS scores determined their significance when comparing graft function at 1-year post transplant,[110] but in the Netherlands, no association with P-PASS following solid organ transplant could be demonstrated.[111] Donation after cardiac death (DCD) is only performed in a few countries worldwide, however, carefully selected DCD is not associated with disparate 1-year graft survival rates when compared with donation after brain death (DBD).[112] Nevertheless, DCD islet yield rates were found to be significantly lower,[113] and DCD pancreas recipients were more likely to develop thrombosis, but this is mitigated when heparin is administered premortem.[114] These limitations result in decreased availability of pancreata and islets available for transplantation, necessitating the consideration of alternative sources of islets and less diabetogenic immunosuppressive agent to avoid multiple islet transplants.

1.4.4 Systemic Immunosuppression

Individuals that undergo islet cell transplantation are subsequently reliant on life-long immunosuppressive medication to prevent rejection of the graft. Despite significant improvement in technique and immune therapy over the past two decades, islet transplantation is currently still a last-resort therapy, only performed on patients whose diabetes cannot be managed by conventional insulin therapy.[8, 115] When all other intensive insulin management

options fail, regardless of technological advances (e.g. continuous glucose monitor with tethered bi-hormonal pumps), islet transplantation is then considered.[115]

Administration of necessary post-transplant anti-rejection drugs suppress the immune response to combat rejection, but simultaneously introduces additional obstacles. Cyclosporine and corticosteroids both contain potent immunosuppressive properties, but bring with them the toxic adverse effects of nephrotoxicity, and diabetogenicity.[116] Tacrolimus, a calcineurin inhibitor (CNI), blocks interleukin-2 (IL2) and prevents T-cell maturation.[117] Use of CNIs commonly results in nephrotoxicity, occurring in 17–50% of kidney transplant recipients that receive CNIs.[117] Sirolimus targets rapamycin, again blocking cellular responses to T-cell and B-cell activation. Medications such as tacrolimus, and mycophenolate mofetil, are toxic to β-cells, inhibiting insulin secretion or preventing proliferation, and consequently impacting graft function.[42, 118, 119] Tacrolimus-induced islet cell toxicity induces new onset diabetes after transplant in 30% of solid organ transplants.[120] Immune-suppressed individuals are also known to be at a higher risk for developing infection or cancer.[121, 122] The "Edmonton Protocol" made significant headway in reducing these harms, through a more potent but less diabetogenic immunosuppressive regime, consisting of sirolimus, low-dose tacrolimus, and the anti-IL2 antibody daclizumab, and avoiding corticosteroids and high doses of calcineurin inhibition.[123] CNI-sparing immunosuppression, either with Belatacept, a T-cell inhibitor, or Efalizumab, a CD11a-specific monoclonal antibody which disrupts T-cell function, has been associated with 40% insulin-independence 10-years post-transplant,[124] suggesting the validity of CNI-avoidance in post-transplant therapy.

Given the adverse effects of systemic immunosuppression and low rates of insulin independence following transplant, the transplant- eligible pool is limited only to patients with

severe and recurrent hypoglycemia unawareness or unstable glucose control despite aggressive medical treatment. For patients that do not experience these complications, it is currently believed that the risks and drawbacks of islet cell transplantation do not outweigh the benefits of insulin independence. Transplant-eligible patients must also maintain a normal BMI, be free of heart disease, and maintain a glomerular filtration rate greater than 80 ml/min/1.73 m^2, as immunosuppressive therapy may decrease kidney function (CITR, 2015) and contribute to renal failure.[105] In order to improve islet transplantation success rates, it is imperative that therapies which minimize the use of immunosuppressive medication are developed.

1.4.5 Hypoxic Injury

Hypoxic injury has been linked to programmed β-cell death, and conversely, the triggering of programmed β-cell death has been linked to hypoxic injury. Earlier studies demonstrated the induction of BCL-2, a family of proteins which include pro- and anti-apoptotic factors, under anaerobic conditions (hypoxia) to prevent apoptosis without the involvement of reactive oxygen species (ROS) at mitochondrial and microsomal membranes.[125], [126, 127] Further studies have linked hypoxia-associated β-cell damage to oxidative stress, as subsequent enhanced mitochondrial respiration results in accumulation of ROS. Peripheral insulin resistance elicits glucose clearance impairment in β-cells, leading to an escalation in ROS production via glycolytic flux increase. Furthermore, influx of intracellular $Ca2^+$ leads to generation of ROS in the mitochondria.[128] Despite attempts to inhibit ROS production, IBMIR, chronic hypoxia, and islet stress during isolation, introduce challenges to the post-transplant preservation of islet mass.[125] Mammals have developed antioxidant defense systems to cope with ROS within the cell, and the three most important include catalase, selenium-dependent glutathione peroxidase 1

(GPX1), and Cu, Zn-superoxide dismutase (SOD1).[129] Their expression is essential in protecting against ROS-induced acute oxidative stress, and furthermore, overexpression leads to dose-dependent increases in protection, however, β-cells express these three enzymes at lower levels than other cells, making them low in antioxidant defence and further susceptible to oxidative stress.[130]

Following transplantation, *in vitro* data has shown that pO_2 in the core of islets is lower than at the surface, tissue, or surrounding medium, and that isolated rat islets exhibit reduced insulin secretion attributed to hypoxia. This can be reversed under normoxic conditions, however, under pro- longed culture conditions, a decline in pO_2 sensitivity occurs by way of depletion of energy (ATP) storage in β-cells, resulting in reduced insulin secretion.[131] Moreover, intraportal islet transplantation into syngeneic mice showed low-oxygenated islet cells (pO_2 < 10 mmHg), as compared to renal subcapsular grafts and native islets, leading to an increased apoptosis rate at the intraportal site when examined one month post-transplantation.[132] Carlsson et al. were also able to demonstrate low oxygenation accompanied by decreased vascular density after one month of transplant in nude mice for both mouse and human islets, despite the rapid revascularization after transplantation, and this low oxygenation decreases the mass of β-cells required in order to obtain insulin independence. A follow up study on human islets with intraportal transplant provided further supportive evidence.[133] In response to hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) associates upstream with the hypoxic response element (HRE) to induce upregulation of hundreds of genes.[134] During normoxic conditions, the von Hippel-Lindau protein (VHL) binds to HIF1-α, which leads to recruitment of E3, a ubiquitin-protein ligase complex, and subsequent ubiquitination and proteosome-mediated degradation of HIF1-α. Comparatively, under hypoxic conditions this

phenomenon does not occur; instead HIF1-α dimerizes with HIF1-β (or ARNT) and accumulates in order to exert transcriptional forces on target genes, and promote physiological pathways that enable cell survival.[134] Of significance, HIF1-α has been associated with an increased expression of lactate de-hydrogenase A (LDHA) in isolated human islets prior to transplantation, and this is maintained after transplantation, leading to poor glycaemic control. Mouse islets exposed to hypoxia in a syngeneic transplant showed pronounced glucose intolerance and failed GSIS driven by a HIF-dependent switch to glycolysis.[135] Early suppression of HIF-1α in transplanted islets results in less β-cell death, therefore minimizing graft failure.[136] Therapeutic interventions to prevent hypoxic conditions, such as the upregulation of HIF-1α, are attractive approaches for maintaining islet mass following transplant.

1.4.6 Islet Insult and Death

Improvements in post-transplant treatments have been improving steadily, however, most combination therapies focus exclusively on preventing graft rejection. Much of the conversation centers on recurring autoimmunity, and prevention of allorejection that occurs via overactivation of the adaptive immune system. However, of more pressing consideration is the IBMIR response that arises immediately following transplant, due to non-specific immune reactions triggered by the exposure of grafted cells to blood.[137] These responses are characterized by the activation of platelets and complement cascades, leading to coagulation of transplanted islets, and damage by leukocytes. Inflammatory molecules and cytokines like IL-1β and TNF-α are released in excess quantities during IBMIR and promote insulitis and β-cell destruction,[138, 139] resulting in the acute destruction of up to 70% of newly transplanted islet cells.[125] Consequently, >70% of patients receive two transplants in order to achieve insulin independence (CITR, 2015).

IBMIR has been linked to the loss of β-cell mass following intraportal islet infusion.[140, 141] Extensive necrosis and apoptosis resulting in 60% loss of islet mass by day 3 was observed following syngeneic transplant of 100 islets into diabetic mice, and consequently normoglycemia was not restored.[141] Thrombin-antithrombin complex, which forms as a response to coagulation following damage, was measured in nine patients, and was found to increase rapidly within 15–60 min after islet infusion, suggesting early islet injury following transplant. Furthermore, patients that exhibited strong initial IBMIR responses showed no significant increase in insulin synthesis after seven days.[142]

The suppression of innate immune reactivity can lead to improved transplant outcomes. For example, inhibition of TNF-α by etanercept, besides prolonged heparin treatment, resulted in the improvement of islet graft of T1DM patients in a clinical trial, following marginal mass transplant from a single donor.[143] Furthermore, *in vitro* culture of islets in low molecular weight dextran sulfate, a heparin-like anticoagulant and anticomplement agent, results in blockage of IBMIR, and reduction of IL-1β and TNFα secretion, which could potentially enhance islet transplantation outcomes by protecting grafts from inflammatory damage.[144] Moreover, in the kidney capsule islet transplantation model, the protective role of anti-apoptotic effect of alpha-1-antitrypsin (AAT), a serine protease inhibitor, in β-cells has been reported, by its ability to improve mitochondrial membrane stability, inhibit apoptosis, inhibit NF-kB activation, modulate the balance of pro-vs anti-inflammatory cytokines, and reduce immune responses.[145] A recent study showed that use of AAT leads to the inhibition of IBMIR and cytokine-induced inflammation in islets following intraportal islet transplantation in mice.[146]

These pro-inflammatory stimuli can trigger islet death through mechanisms such as extrinsic apoptosis. Apoptosis is a form of programmed and regulated cell death, which is

stimulated by extracellular stresses and initiated by intracellular signaling or extracellular ligand binding. This regulated form of cell death is an important contributor to human islet cell loss following isolation.[147] The process most commonly begins with the binding of TNF-α to its receptor, tumour necrosis factor receptor 1 (TNFR1), which is found ubiquitously across all cell types.[148] Apoptotic death processes are mediated by caspase-8, which is cleaved and activated by RIPK1 (receptor interacting serine/threonine-protein kinase 1) and FADD (Fas-associated protein with death domain). Asides from apoptosis, caspase-8 also has a role in cytoskeleton remodelling[149] and lymphocyte and natural killer cell activation.[150] Apoptosis is the primary contributor of programmed cell loss in isolated islet cells, as measured through immunohistochemical analysis for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and DNA fragmentation.[147]

Comparatively, necrosis was conventionally believed to function as an unregulated cell death process, occurring as a response to environmental stresses such as heat or osmotic shock, mechanical or other physiological stress, or viral infections. Subsequent damage of the cell causes cell and organelle swelling (oncosis), and the intracellular contents are released into the extracellular space, triggering an inflammatory response. Necrosis and apoptosis are equivalent contributors to islet loss - following isolation, islets have been observed to have a zone of central necrosis (correlated with a decrease in viability and GSIS) and an increase in apoptosis.[151] Grafts harvested from STZ-induced diabetic mice transplanted with syngeneic islets indicated extensive islet necrosis at three days post-transplant.[152] Conventional thought paid little attention towards preventing necrosis in comparison with apoptosis, as it was believed that unregulated cell death could not be controlled, despite its high impact on post-transplant cell death.

However, it has recently come to light that there are multiple pathways of necrosis, with some subtypes susceptible to cellular control and regulation. Regulated necrosis occurs following genetically controlled cell death, leading to oncosis. As these mechanisms are controlled at the cellular level, they provide an opening to target necrosis. Many subtypes have been identified, including necroptosis, ferroptosis, pyronecrosis, and parthanatos.[153] These regulated, non-apoptotic cell death pathways exhibit distinct biochemical characteristics but have yet to be fully characterized within islet transplantation.[153] Necroptosis was first uncovered in 2005,[154] and is the best understood process thus far. Necroptosis is a caspase-independent process, and can be initiated through release of ROS, the binding of TNF-α to TNFR1, calcium overload,[155] or TLR3/TLR4 activation.[156]

Necroptotic death occurs in absence of caspase 8. cFLIP, an anti- apoptotic protein binds to and inhibits caspase 8, which allows for recruitment and binding of RIPK3 (receptor interacting protein kinase 3) to RIPK1. The two are activated via *trans*-autophosphorylation, causing formation of the necrosome, which then further phosphorylates mixed lineage kinase domain-like protein (MLKL), the third component of the necrosome. MLKL will oligomerize, and enter the plasma and nuclear membrane, creating membrane-disrupting pores.

All cells undergoing necroptosis share similar features to cells undergoing necrosis – the cell and organelles will swell, the plasma membrane ruptures, and the intracellular contents, including DAMPs, leak into the extracellular space, triggering an inflammatory response. In vitro data suggests that this process takes at least 3h.[157] HMGB1 (high mobility group box 1 protein) is the most commonly released DAMP molecule following necroptosis. It has been shown that the presence of extracellular HMGB1 regulates cell survival or necroptosis by activating or repressing the nuclear factor kappa B (NF-kB) pathway.[158] RIPK1 can also

induce proinflammatory cytokines independently of RIPK3 and MLKL, through TNF-α -driven NF-kB activation.[158]

Necroptosis has not previously been examined in islets, however, following hepatic ischemia reperfusion injury (IRI), RIPK1 and RIPK3 expression is increased, and this increased expression is accompanied by the formation of the RIPK1/RIPK3 complex.[159] RIPK3 has also been identified as an important mediator of necroptosis in various whole organ mouse transplant models. For example, delayed graft rejection was observed when hearts from RIPK3-deficient mice were transplanted in immunosuppressed recipients, in comparison with mice receiving wild-type hearts.[160] RIPK3 deficiency was found to relieve skin[161] and intestinal[162] inflammation, and potentially could assist in mitigating IBMIR. Correspondingly, inhibition of necroptosis following islet transplantation may also prove to be beneficial.

1.5 Targeting Cell Death Pathways

Therapeutic targeting has the potential to protect islet viability by blocking inflammatory and cell death pathways to dampen the degree of IBMIR. Immunomodulatory therapies and anti-inflammatory regimes serve to increase the presence of immunoregulatory cytokines or inhibit the presence of proinflammatory cytokines. Apoptosis and regulated necrosis are two cell death pathways that can be controlled through specific inhibitors, thereby maintaining islet cells following transplantation. Minimizing acute islet death in the immediate post- transplant period, and thereby improving engraftment efficacy, is of top priority.

1.5.1 Inflammation

Inflammatory responses within the body can be combatted through multiple lines of approach. TNF-α is a key regulator of immune cells and inflammation; consequently, TNF inhibitors can be used in suppression of this inflammation. Between 1999 and 2002, TNF-α inhibitors were utilized in 11.8% of transplants, and between 2007 and 2010, TNF-α inhibitors were administered in 34% of transplants. Different TNF-α inhibitors display various properties. Entanercept (ETA) with the GLP-1 receptor agonist exenatide has been shown to permit increased insulin independence and lowering of HbA1C, in comparison with no ETA treatment.[163, 164] ETA, when used in combination with a T-cell depletion regimen, show success rates of islet transplants which are comparable to success rates of whole pancreas transplants, standing at approximately 50% after 5 years.[47] Combination therapy with an interleukin-1 receptor antagonist (ANA) results in decreased presence of pro-inflammatory cytokines, and therefore decreased IBMIR.[165] 2015 data from CITR also indicated that use of T-cell depletion and/or TNF-α inhibitors is associated with improved post-transplant outcomes, although these registry data were unable to definitively confirm causative factors.

Low molecular-weight dextran sulfate (LMW-DS) was shown to reduce the presence of IBMIR in a non-human primate model of islet cell transplantation[166] through inhibition of the complement cascade and coagulation system, and prevention of cell-cell adhesion. A phase II clinical trial conducted by Uppsala University in Sweden, in conjunction with the Clinical Islet Transplantation (CIT) consortium, indicated the safety and efficacy of LMW-DS, comparable to the gold-standard treatment with heparin.[167] Unlike heparin however, LMW-DS confers a lower risk of uncontrolled bleeding, and promotes intrahepatic islet engraftment.[168]

IBMIR reduction has also been achieved through administration of Complement 5a inhibitory peptide (C5aIP) and the thrombin inhibitor Melagatran. C5a, a chemotactic agent, acts as an inflammatory peptide, releasing a range of cytokines, and stimulating the innate immune response. Prevention of this response through C5aIP, which specifically suppresses the thrombin-antithrombin complex formation, attenuates crosstalk between the complement and coagulation cascades, suppressing the expression of tissue factors on granulocytes in recipient livers.[169] Melagatran was found to reduce IBMIR in a dose-dependent manner.[170] Minimizing IBMIR through reduction of inflammatory responses and inhibition of the complement cascade could potentially improve islet cell engraftment.

1.5.2 Apoptosis

Apoptosis can be triggered via intracellular or extracellular cues, both initiating the apoptotic cascade. Therapeutic targeting primarily aims to inhibit caspase activation, with the goal of preventing the downstream effects of this cascade. Caspase-8 can be inhibited by a pan-caspase inhibitor, Z-VAD-FMK, or cFLIP mimetics, which would cause all TNF-α initiated cell death events to be shunted towards necroptotic events. cFLIP overexpression has been observed

to prevent cytokine- mediated apoptosis in a murine β-cell model,[171] however, similar results were not observed in 3–6 week old NOD mice.[172] Caspase inhibition through the pan-caspase inhibitor Z-VAD-FMK prevented TNF-α induced apoptosis at 24 h, but hepatocyte death via necrosis was observed at 48 h. Pan-caspase inhibitors such as F573, IDN-6556, and EP1013 have been shown to improve graft success following intraportal islet infusion.[173-175]

Activation of NF-kB within β-cells, leading to ROS generation and upregulation of proinflammatory genes, can also trigger pro-apoptotic mechanisms. An endogenous inhibitor of NF-kB, A20, functions to prevent both extrinsic and intrinsic apoptosis signaling via the inhibition of TNF-induced apoptosis.[176] Heme-oxygenase-1 (HO-1) primarily plays a role in iron homeostasis, but also acts as an anti-inflammatory agent and anti-apoptotic gene. Expression of HO-1 induced through culture in ferriprotoporphyrin IX chloride and cobaltic protoporphyrin IX chloride prior to transplantation, was shown to protect islet cells from apoptotic death.[177] However, the translation of anti-apoptosis therapy in clinical islet transplantation has yet to manifest in preferential outcomes.

1.5.3 Regulated necrosis

Necroptosis can be prevented at multiple points throughout the pathway. Necrostatin-1 (nec-1) acts as an allosteric inhibitor of RIPK1 and RIPK3 with high specificity, preventing necroptosis. Nec-1 is not detrimental to healthy cells; it does not negatively alter ATP levels, cell shape and size, plasma membrane integrity, or cell proliferation. Although other inhibitors are in development, nec-1 is unique in its ability to specifically block RIPK1 and RIPK3[178] to regulate necroptosis.

Nec-1 prevents the necroptotic death of TNF-α-treated cells,[179] even in the absence of z-VAD, by protecting plasma membrane integrity.[180] The same study established that treatment with nec-1 did not result in observable apoptotic markers or apoptotic morphology, suggesting that nec-1 does not have an impact on apoptosis, but does selectively inhibit necroptosis. Nec-1 also increases insulin secretion, upregulates GLUT2 transporters, and doubles α and β-cell composition following 7 days culture of pre-weaned porcine islets.[181] Treatment of Nec-1 15-min pre-ischemia or 15-min postreperfusion reduced renal damage and preserved renal function as measured via urea serum creatinine at 48h.[182] Four days co-culture of pre-weaned porcine islets in 100 μM nec-1, but not 0, 25, 50, or 200 μM nec-1, resulted in significantly higher islet recovery, GLUT2 expression in β-cells, and insulin secretory capacity.[181]

NF-kB-mediated inflammatory processes and pro-inflammatory molecules like TNF-α, IL-1b and IL-6 were decreased in the presence of nec-1, in a mouse model.[183] Increased expression of A20, a ubiquitin editing protein, modulates NF-kB through regulation of RIPK1 levels, reducing neonatal porcine islet inflammation, increasing post-transplant function, and promoting cell survival.[184] Comparatively, another study found that treating T2DM mice with nec-1, or depleting MLKL levels prevented insulin resistance, but did not have an impact on inflammation or cell death.[185]

However, nec-1 also inhibits indoleamine 2,3-dioxygnase, which contains both innate and adaptive immune functions, and is activated in the presence of inflammation,[186] and is toxic at low concentrations. An alternative, necrostatin-1s (nec-1s) has a longer half-life and is more stable than nec-1, as it only targets RIPK1. It functions by locking RIPK1 into an inactive

configuration and prevents its phosphorylation and ubiquitination.[187] Nec-1s also lacks the IDO inhibitory activity and concentration-dependent toxicity *in vivo*.[186]

As RIPK1 is involved in both apoptosis, necroptosis, and NF-kB- related pathways, inhibition of RIPK3, and therefore sole inhibition of necroptosis, may prove to have higher therapeutic relevance. The use of RIPK3 specific inhibitors such as ponatinib and pazopanib will block necroptosis but not apoptosis.[188] Similarly, GSK′840, GSK′843, GSK′872, and GW392 in low concentrations will inhibit RIPK3 and prevent necroptosis in low concentrations,[189] however, in high concentrations may also induce apoptosis by activating caspase-8.[190] Inhibition of RIPK1 and RIPK3 by nec-1 partially inhibited TNF-α/ZVAD induced necrosis in hepatocytes, but genetic deletion of RIPK3 in mice exacerbated liver injury following treatment with LPS and ZVAD, suggesting that RIP3-mediated death may be context and tissue dependent.[191]

The third molecule directly involved in the necroptosis pathway, MLKL, also offers a potential avenue for targeting. The MLKL inhibitor necrosulfonamide prevents necroptosis *in vitro*[192] but cannot be tested in murine models as necrosulfonamide is specific to human MLKL.[193] Treatment with the small-molecule inhibitor PK68 has been observed to prevent TLR3 and TLR4 mediated necroptosis in bone-marrow derived macrophages by acting specifically and independently on RIPK1 and RIPK3, but PK68 does not act on MLKL.[192] Targeting the inhibition of regulated cell death may aid in elucidating critical factors that govern islet cell death and survival post-transplant.

1.5.4 Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) homeostasis is defined by the equilibrium that occurs between the cellular demand for protein synthesis, and the ER folding capacity to produce these proteins.[194] Stresses to the system, such as the ones that occur in β-cells during times of extreme blood glucose fluctuations, often disrupt this homeostasis and lead to accumulation of unfolded and misfolded proteins within the ER lumen.[195] Both acute (1-3 hours) and chronic (over 24 hours) high glucose conditions have been shown to induce activation of the ER transmembrane protein Inositol Requiring 1 (IRE1) within both mouse and rat islets.[196] Prolonged activation of IRE1 leads to insulin mRNA degradation, induction of pro-apoptotic effectors, and β-cells dysfunction and death.[197] Consequently, it is believed that ER stress contributes to the inflammation observed within the islet during diabetic disease processes.

Furthermore, it is possible that this process can be affected by RIPK3. As described above, RIPK3 functions in regulated necrosis to induce cell death. However, it can also regulate inflammation independent of its role in necroptosis. It is known that RIPK3 can activate NF-κB, which is chronically active in inflammatory disease processes, and leads to the expression of pro-inflammatory cytokines and chemokines.[198] Activation of RIPK3 has been observed to occur in mouse islets and human islet grafts in diabetic mice, following ER stress.[199] Taken together, it is possible that inhibition of ER stress, or inhibition of RIPK3, may prevent the associated downstream effects of islet inflammation.

1.6 Objectives, Outlines, and Hypotheses

The overarching objective of this thesis is to understand the role of apoptosis and regulated necerosis in β-cell death, and to determine whether β-cell death can be prevented by inhibiting apoptosis and regulated necrosis. Both apoptosis and necroptosis pathways in β-cells have been explored previously, and inhibition of apoptosis has been studied extensively in the context of β-cell death following transplantation. However, necroptosis inhibition, and inhibition in conjunction has not been previously explored, indicating a clinically relevant, novel avenue to pursue the prevention of β-cell death.

This objective can be achieved by studying the effects of necroptosis and apoptosis inhibition in a mouse insulinoma (MIN6) immortalized cell line which retains its ability to secret insulin in response to glucose, in human islets *in vitro*, and in human islets transplanted into diabetic mice. To begin, the feasibility of inhibiting necroptosis will need to be established, and this can be done by targeting RIPK1 via siRNA. Confirmation of knockdown via western blot, and subsequent vitality and GSIS studies will establish the phenotypic and functional differences between wild-type and siRIPK1 MIN6 cells. Similarly, pharmacological inhibition can be performed using RIPK1, RIPK1/RIPK3, and pan-caspase inhibitors alone and in combination, using Nec1, Nec1s, ZVAD, respectively in both human islets and MIN6 cells. Confirmation of the knockdown of RIPK1 can be performed via western blotting. Functional studies can be performed to determine whether application of the inhibitors for 24, 48, or 72 hours prior to assay results in differences between cellular function under basal and stressed conditions. Furthermore, understanding of the impact of these inhibitors will ensure that the insulin-producing capacity of the MIN6 cell or human islet is preserved following application of inhibitors and in the face of cell death initiation.

Next, the effect of transplanting a marginal mass of human islets treated with inhibitors for 24 hours into streptozotocin induced diabetic immunodeficient B6.129S7-Rag1^{tm1mom}/J mouse model, to ascertain whether inhibition of apoptosis and necroptosis will permit improved glycemic control and improved glucose clearance. Streptozotocin is a glucose analog which enters β--cells via the GLUT 2 transporters and induces DNA alkylation and production of free radicals, resulting in cell damage and death. Blood glucose can be monitored twice weekly to track rates of euglycemia, and an intraperitoneal glucose tolerance test can be performed to observe glucose clearance rates. Observing rates of reversal of diabetes in a mouse model will permit for the best understanding of the stresses that islets undergo following transplant into human patients.

It is hypothesized that Nec1 will successfully inhibit both RIPK1 and RIPK3 and Nec1s will inhibit RIPK1, thereby inhibiting necroptosis, and ZVAD will inhibit apoptosis in MIN6 cells and human islets. Following cell death initiation *in vitro*, inhibition of necroptosis and apoptosis either alone or in combination will lead to improved cell vitality, GSIS, and cell function as compared with controls. Finally, following marginal mass transplant of human islets into diabetic mice, islets cultured in necroptosis and apoptosis inhibitors either alone or in combination will result in mice with improved glycemic control and glucose clearance.

1.7 Summary

T1DM is an increasingly prevalent concern around the world. Insulin replacement therapy remains the mainstay treatment for management of T1DM, and this has been paired with advances in the types of insulin, and in the technological treatment options which aim to approximate physiological glucose monitoring and release. Despite these advances, there is a minority of patients for whom these options are not sufficient to maintain appropriate glycemic control – current therapies are effective in preventing SHEs in 50–80% of patients with IAH and SHEs, which still means that 20-50% of patients aren't able to manage with exogenous insulin administration alone. This often necessitates an islet transplant; however, the efficacy of this procedure leaves significant room for improvement.

Following a transplant, significant cell death occurs acute period - approximately 80% of islets will die within 24 hours after transplant. A significant proportion of death occurs due to apoptosis and necrosis, however, there has been limited focus placed in controlling their effects, particularly in the case of necrosis. The goal of this project is to better characterize the role of regulated necrosis and apoptosis in islet death, and ascertain whether inhibition of these pathways, whether alone or in combination, leads to reduced cell death in the post-transplant period. Improved understanding of islet physiology and cell death may ultimately lead to improved efficacy of transplant in human patients, resulting in better engraftment rates, lower doses of islets required per transplant, and increased availability of transplant to a broader range of T1DM patients.

CHAPTER 2: EXAMINING THE ROLE OF NECROPTOSIS IN B-CELL LOSS FOLLOWING ISLET TRANSPLANTATION

2.1 Introduction

Islet transplantation is the best way to recapitulate physiological insulin delivery. However, despite allowing for significant improvement in management of T1DM, it is utilized as a last resort therapy, due to a multitude of factors. Firstly, a shortage in the supply of islets naturally acts as a limiting factor. Secondly and more significantly, the lifelong requirement of immunosuppressive medication following transplant to prevent graft rejection introduces significant burden to the patient[71], and reduces the availability of islet transplant to only the most brittle of patients with T1DM. Thirdly, high rates of graft attrition result in low rates of single procedure insulin independence. Consequently, only 50% of patients are insulin independent after 5 years following transplant[200], and 73% of patients require a second transplant to achieve true insulin independence. Significant efforts have been invested in understanding and overcoming long term graft rejection, however, islet graft loss can also be attributed to the instant blood mediated inflammatory reaction (IBMIR) that arise immediately following transplant, due to non-specific immune reactions triggered by the exposure of grafted cells to blood.[137] The result is that 24 hours after transplant, 80% of newly transplanted islet cells will die. Minimizing acute islet cell death in the immediate post-transplant period is of top priority.

Cell death in this period of time is mediated in multiple different ways. The primary form of islet death occurs via apoptosis, a programmed and regulated cell death, stimulated by extracellular stresses.[201] Cells undergoing apoptosis will shrink and fragment, followed by removal of the cell particles by phagocytic cells. This blebbing process does not trigger the immune response, as stimulatory factors are not secreted or produced. This pathway is well established in islet transplant dysfunction, however, despite the provision of adjuvant systemic

inhibitive therapy alongside pre-treatment of islets prior to transplant, preventing or controlling apoptosis alone in the post-transplant period is insufficient in promoting β-cell survival. A secondary pathway of cell death, necrosis, can therefore also be considered. Necrosis is an unregulated cell death process, occurring as a response to environmental stresses such as heat or osmotic shock, mechanical or other physiological stress, or viral infections.[202] This damage of the cell causes cell and organelle swelling, and the intracellular contents will be released into the extracellular space, triggering an inflammatory response, and attracting immune cells. As an unprogrammed form of cell death, necrosis is considered to be difficult to control, however, a subtype of necrosis, necroptosis, shares features of both necrosis and apoptosis, and provides an avenue to target necrosis.

In necroptosis, receptor interacting protein kinase (RIPK) 1 and 3 come together and activate each other via trans-autophosphorylation, causing formation of the necrosome, which then further phosphorylates mixed lineage kinase domain-like protein (MLKL), the third component of the necrosome.[202] MLKL will oligomerize, and enter the plasma and nuclear membrane, creating membrane-disrupting pores. RIPK1/3 inhibition has been performed in experimental disease models – of most relevance, ischemia reperfusion in the kidney,[203] but also in the brain and heart, as well as in retinal cell death, and systemic inflammatory responses.[204] It is difficult to understand the proportion of cell death that occurs through each pathway. Thus far, there has not been a lot of research in understanding the role of regulated necrosis in islet cell graft dysfunction, however, it is possible that inhibition of the formation of the necrosome complex could prevent necroptosis-related islet death.

This project aims to understand the function of apoptosis and necrosis inhibition in islet graft dysfunction. This was accomplished by examining inhibition of necroptosis and apoptosis

in β-cells via genetic knockdown and pharmacologic inhibition, and ascertaining whether this inhibition will improve islet cell survival and function. Intervening in either of the apoptotic or necroptotic processes might allow for the inhibition of islet death, and consequently, better allow for graft function and survival.

2.2 Materials and Methods

2.2.1 MIN6 Cells:

MIN6 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA, USA) containing 25mM glucose and supplemented with 15% fetal bovine serum (Gibco), B-2-mercaptoethanol, and penicillin-streptomycin (Lonza Bioscience, Basel, CHE). Cells were kept in a humidified atmosphere at 37°C with 5% CO_2 and passaged with Trypsin-EDTA (0.05%, Gibco) on a weekly basis once confluency of 80% was achieved. Cells were changed with complete medium every 48-72h.

To perform cell viability, insulin secretion, and oxygen consumption rate assay, and western blotting, cells were seeded in 6- or 12-well plates and grown to a confluency of 80%. Necroptosis and apoptosis inhibitors were introduced to culture media (Necrostatin-1 at 100µM, Necrostatin-1s at 100µM, Necrostatin-i at 100µM, ± Z-VAD-FMK at 20µM), and cells were incubated for 24- or 48-hours at 37°C with 5% CO_2. Cell death was induced via application of 400µM H_2O_2 for 2 hours, at 37°C with 5% CO_2.

2.2.1.1 Cell Membrane Integrity Assay:

MIN6 cells were removed from 12-well plates. 100uL of media containing MIN6 cells was mixed with 100uL of trypan blue (Gibco) to assess for cell viability via dye exclusion. 10uL of the mixed solution was loaded onto a hemocytometer, and counts were manually performed in triplicate. Membrane integrity was assessed as a proxy for cell vitality.

2.2.1.2 Oxygen Consumption Rate Assay:

MIN6 cells seeded in 6-well plates were removed via trypsin-EDTA from each well. OCR was measured in duplicate using a fiber-optic sensor oxygen monitoring system (Instech Laboratories, Plymouth Meeting, PA) which quantifies the decrease in oxygen partial pressure over time. OCR (nmol/min) was standardized to the amount of DNA (mg) in each chamber, and DNA was quantified via the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR) as per manufacturer's instructions.

2.2.1.3 Western blotting:

Following 48-hour incubation in 12-well plates, MIN6 cells were washed twice with cold PBS. 100µL lysis buffer (1% 1M Tris pH 8.0, 3% 5M NaCl, 1% Triton X-100, 1 Protease Inhibitor Cocktail tablet (Roche), 0.5% 200mM sodium orthovanadate phosphatase inhibitor) was added to each well on ice for 20 minutes, and cells were scraped from plate, added to microcentrifuge tubes, and sonicated briefly. Cells were then spun down for 10 minutes at 12,000rpm at 4°C, and supernatant was frozen in liquid nitrogen at -80°C until further use.

Protein concentration was determined via Pierce BCA Protein Assay Kit (Waltham, MA, USA), as per manufacturer's instructions.

Samples were denatured in NuPAGE LDS Sample Buffer 4x (Thermo Fisher) at 95°C for 8 minutes. Equivalents amount of protein was loaded onto commercially produced pre-cast 10% gels (Bio-Rad, Hercules, CA) and fractioned via electrophoresis. Gels were transferred to nitrocellulose membranes via semi-dry blotting apparatus at 12V for 90 minutes. Membranes were blocked in 1% BSA in PBS for 30 minutes at room temperature. Primary antibody was specific for RIPK1 (1/1000, Cell Signalling Technologies, Danvers, MA, USA), p-RIPK1

(1/1000, Thermo Fisher), and β-actin (1/1000, Thermo Fisher) Membranes with primary antibodies were incubated overnight at $4^{O}C$. Blots were then washed in PBS 3x15 minutes and incubated in fluorescent goat anti-rabbit (1/10,000, Invitrogen) or goat anti-mouse (1/20,000, Invitrogen) secondary antibody at room temperature for one hour. Membranes were then washed again with PBS 3x15 minutes, and blots were developed using the LI-COR Odyssey scanner and software.

2.2.1.4 siRNA:

At time of passage, MIN6 cells were co-transfected with lipofectamine 2000 and siRIPK1 in OptiMEM media. Briefly, siRNA was added to Opti-MEM media and blended gently. Opti-MEM was also used to dilute the Lipofectamine 2000 reagent. The two were added in equal concentrations within the 6-well plate, and allowed to interact for 5 minutes at room temperature to form the siRNA-lipofectamine complex. MIN6 cells suspended in serum- and-antibiotic- free medium, at a concentration of $1.0x10^6$ cells/well were added to each well, and grown at 37°C with 5% CO_2 without changing the medium. After 72 hours of transfection, cell vitality staining, GSIS, and Western Blotting was performed as described above.

2.2.2 Human Islets:

Human islets were obtained from the Alberta Diabetes Institute (ADI) Islet Core, through the Human Organ Procurement and Exchange Program (**Table 1)**. Human islets were cultured in CMRL media (Corning-Costar Corporation, Cambridge, MA, USA), 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). Islets were divided and cultured in Necrostatin-1 (MilliporeSigma, St. Louis, MO, USA), Necrostatin 1s (MilliporeSigma), Necrostatin-inactive (Cayman Chemical Company, Ann Arbor, MI, USA), GSK'872 (Sigma), and Z-VAD-FMK (R&D Systems, Minneapolis, MN, USA).

Prior to performing insulin secretion assay, oxygen consumption rate assay, and marginal mass transplantation, islets were divided across 6-well culture plates and pre-cultured in various inhibitors. Necroptosis and apoptosis inhibitors were introduced to culture media (Necrostatin-1 at 100µM, Necrostatin-1s at 100µM, Necrostatin-i at 100µM, ± Z-VAD-FMK at 20µM), and islets were incubated for 24, 48, or 72 hours at 37°C with 5% CO_2. Cell death was induced via application of 400µM H_2O_2 for 2 hours, at 37°C with 5% CO_2 unless otherwise noted.

Representative islet samples were taken for counting in triplicate prior to and following culture with inhibitors. Human islets were differentiated from acinar tissues and other impurities by Dithizone, which stains the zinc granules present in the β-cell red. Islets of different sizes were volumetrically adjusted to islet equivalents (IEQ) through counting representative samples of islets using a light microscope and grid. A single IEQ has a mean diameter of 150µM, and contains approximately 1000 cells. Representative aliquots were taken to create each replicate for experimentation.

Table 1. Characteristics of human islets, received from 14 independent human donors. 41 mice were transplanted with marginal doses of islets for long term study and were assessed for post-nephrectomy hyperglycemia before inclusion in this study. 18 mice were transplanted with marginal doses of human islets for the purposes of examining inflammation in the acute post-transplant period.

Human Islet Batch Number	Donor Age (years)	Sex	BMI	Purity (%)	Islets utilized for
H2349	69	F	30.1	40	Long term (n=2)
R389	65	F	24.4	75	Long term (n=3)
R403	35	F	37.2	95	Long term (n=4)
H2373	59	M	25.6	37.5	Acute engraftment (n=6)
H2387	43	F	31.1	40	Long term (n=7)
R408	39	M	27.5	80	Long term (n=5)
R410	59	F	26.4	80	Long term (n=7)
R412	42	M	29.9	85	Long term (n=4)
R413	41	M	33.1	90	Long term (n=5)
R419	70	M	31.5	80	Acute engraftment (n=6)
R420	55	M	23.5	85	Acute engraftment (n=6)
R430	49	M	24.4	90	Long term (n=4)

2.2.2.1 Glucose Stimulated Insulin Secretion Assay:

Following 24- and 48-hours culture in the inhibitors, 500 human islets per replicate were washed in a glucose free medium for 3 washes of 5 minutes apiece, followed by incubation in RPMI-1640 (Sigma Aldrich) containing low glucose (2.8mmol/L) for one hour at $37^{O}C$. The supernatant was removed and stored at $-20^{O}C$, and islets were then incubated in RPMI-1640 containing high glucose (24.4mmol/L) for a second hour at $37^{O}C$. The supernatant was again removed and stored at $-20^{O}C$.

Insulin secretion was standardized to total insulin content. A 200μL suspension of cells and media was removed from each sample, in triplicate. The cell suspension was centrifuged at 1000rpm for 1 minute, supernatant was removed, and 1mL of azol (11.4% v/v glacial acetic acid, 0.25% BSA) was added. Samples were stored at -20°C, sonicated, lyophilized, and resuspended in FSA buffer (10mM monosodium phosphate, 150 mM NaCl, 0.05% BSA) prior to quantification.

Insulin was measured using a mouse/rat insulin kit as per manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD, USA).

2.2.2.2 Mitochondrial respiration:

An Extracellular Flux Analyzer XF24 (Seahorse Bioscience, North Billerica, MA, USA) was used to monitor oxygen consumption. Following 72 hours incubation in inhibitors, including GSK'872, and 2 hours exposure to H_2O_2 as described above, 70 human islets were handpicked in triplicate and seeded into wells containing 500 μL assay medium. Basal respiration was measured in 2.8mM glucose media for 30 minutes. Islets were then sequentially exposed to 16.8mM glucose, 5μM Oligomycin A, 3μM FCCP and 5μM Antimycin A/rotenone to inhibit

each complex in the electron transport chain independently of one another. Separately, cell death was also initiated via 2-hour exposure to 5µM thapsigargin.

2.2.3 <u>*Marginal Mass Transplantation*</u>

2.2.3.1 Induction of diabetes in mice

Male and female B6.129S7-Rag1^{tm1Mom}(B6/Rag$^{-/-}$) mice (Jackson Laboratory, ME, USA) were used as recipients for human islet studies. Mice were maintained in a pathogen-free, climatized environment, and given free access to pelleted food and water containing Novo-Trimel. 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 (AUP00003230, AUP00002977). Equivalent numbers of male and female mice were utilized.

Prior to transplantation, recipient mice were rendered diabetic by chemical induction, via intraperitoneal streptozotocin (Sigma, St. Louis, MO, USA), dissolved in acetate buffer (pH 4.5), at 180 mg/kg (male) and 170 mg/kg (female). Blood samples were obtained from the tail for glucose measurement (OneTouch Ultra2 Glucose Meter) twice weekly. Diabetes was confirmed when two consecutive readings of above 15mmol/L were obtained.

2.2.3.2 Transplantation:

Following 24h culture with inhibitors, human islets were counted and transplanted under the left kidney capsule at a marginal islet dose of 500 islet equivalents per diabetic recipient. Grafts were aspirated into polyethylene tubing, pelleted via centrifugation, then placed under the left kidney capsule with the help of a micromanipulator syringe. Prior to recovery, all recipients received a 0.1mg/kg subcutaneous bolus of buprenorphine, and mice were monitored for acute

hypoglycemic events which was treated through intraperitoneal injection of glucose. 100µL of 50% dextrose was injected if mice were hypoglycemic, and mice were reassessed hourly.

2.2.3.3 Follow-up of long-term graft studies:

Non-fasting blood glucose measurements were made twice weekly following transplant. Graft function and reversal of diabetes was defined as two consecutive readings under 11.1mmol/L which was maintained until study completion. Intraperitoneal glucose tolerance testing was performed at 30 days post-transplant to assess metabolic capacity. Mice were fasted overnight (12h) before receiving intraperitoneal glucose (3g/kg). Blood glucose, obtained from sampling at the tail, was measured at baseline (time 0), then at 15-, 30-, 60-, 90-, and 120-minutes post-injection. Blood glucose area under the curve was analyzed between transplant groups. At 60 days post-transplantation, all mice underwent a survival nephrectomy of the graft-bearing kidney to confirm graft-dependent euglycemia. Nephrectomized animals were subsequently monitored for one week to confirm return to a hyperglycemic state. Graft-bearing kidneys were collected and fixed in 10% paraformaldehyde.

2.2.4 Statistical Analysis:

Statistical analysis was conducted using the GraphPad Prism 9 software (GraphPad Software, La Jolla, Ca, USA). Data are represented as mean ± standard error of mean (SEM). All data groups represent three separate cell passages or human islet donors, assayed in duplicate, unless otherwise noted. In vivo and in vitro data analysis between treatment groups was conducted via two-way ANOVA with Tukey's post-hoc test for multiple comparisons for the

analysis of variances between groups. Kaplan-Meier survival curves were compared via log-rank statistical testing (Mantel-Cox). P less than 0.05 was considered significant.

2.3 Results

2.2.5 Characterization of inhibition of apoptosis and necroptosis in MIN6 cells

To better understand the role of necroptosis in islet cell death, we first confirmed the ability to decrease RIPK1 expression in MIN6 cells. Incubation of MIN6 cells in siRIPK1 for 72 hours indicated successful knockdown of RIPK1, as suggested by western blot (**Figure 1A**). Membrane integrity staining with trypan blue was performed to quantify differences in vitality following siRNA knockdown, and following the induction of cell death via H_2O_2. In the absence of H_2O_2, cell vitality remains consistent between control and siRNA conditions (88.00% ± 0.42 vs 92.44% ± 0.63, $p<0.001$) however, following H_2O_2 administration, significantly improved vitality is observed in the siRNA condition as compared with the control (63.35% ± 1.88 vs 10.84% ± 0.14) (**Figure 1B**). Furthermore, a GSIS assay indicated similarly: comparison of the control high glucose condition (7221 pmol/L ± 543.2) to the control low condition (3146 pmol/L ± 250.2, $p<0.01$) and to the post- H_2O_2 control condition (4560 pmol/L ± 389.3, $p<0.05$) indicated reduced glucose stimulation, however, comparison to the siRNA group with (6794 pmol/L ± 1152) and without H_2O_2 administration (7092 pmol/L ± 238.1) indicated conserved function (**Figure 1C**), suggesting the protective effect of siRIPK1 on MIN6 cells.

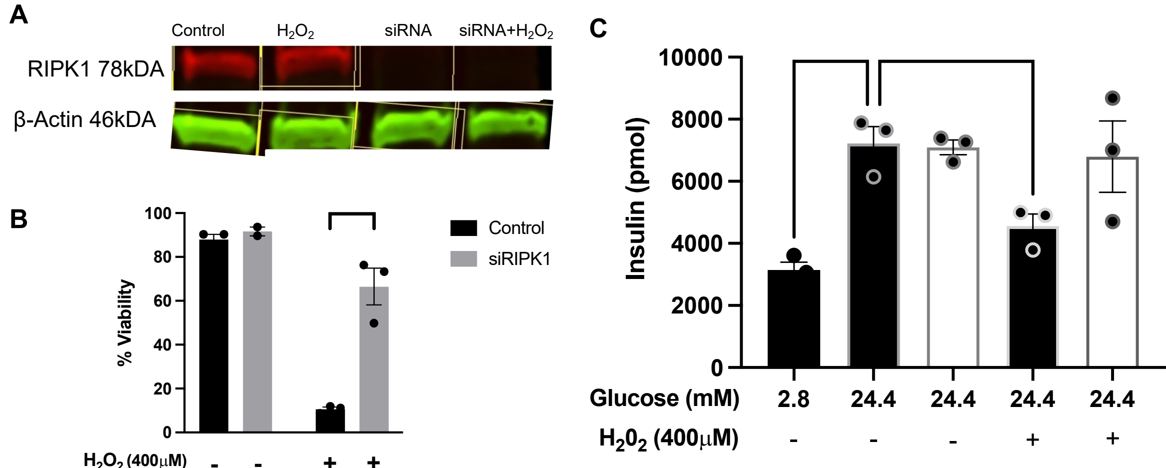


Figure 1. 72-hour incubation of MIN6 cells in siRIPK1, followed by subsequent western blot and vitality and GSIS assays confirm knockdown of RIPK1 and corresponding protection of MIN6 cells from ROS-induced cell death. **A)** Western blot of RIPK1 and B-actin (housekeeping gene) indicates successful loss of RIPK1 in both the control and H_2O_2 conditions. **B)** Membrane integrity staining with trypan blue to examine cell vitality indicates significantly increased vitality in the siRIPK1 group (n=3) as compared with control (n=3), following H_2O_2 administration (unpaired t-test, mean ± SEM, ***p<0.001). **C)** GSIS assay indicates comparable function between pre- H_2O_2 administration control (n=3) and post- H_2O_2 administration in the siRNA group (n=3), with significantly reduced function in the pre- H_2O_2 administration control group (unpaired t-test, mean ± SEM, *p<0.05, **p<0.01).

Pharmacological inhibitors of regulated cell death were then examined, as they provide a more clinically relevant avenue to preventing cell death. MIN6 cells were cultured for 24 or 48 hours in necroptosis and apoptosis inhibitors (Nec1, Nec1s, Nec-i, ZVAD), and combinations of these inhibitors, to better understand their effect in cell death inhibition. Following culture, cell death was initiated using H_2O_2. A dose-response curve was constructed to determine optimal H_2O_2 concentration following 2 hours of culture (**Supplemental Figure 1**), and 400µM was used in all experimentation henceforth. Various cell viability and function metrics were assessed sequentially.

Cell vitality was first examined using a membrane integrity stain. In the control conditions, similarly high levels of vitality were seen regardless of inhibitor application in both the 24- and 48-hour timepoints (**Figure 2A, 3A**) However, following administration of H_2O_2, disparate levels of vitality are observed. In the 24-hour timepoint, significant improvements in vitality are seen between Nec-i (19.3% ± 4.8), the inactive control, and ZVAD (39.8% ± 6.2, $p<0.01$, Nec-1/ZVAD (42.5% ± 2.4, $p<0.01$), Nec-1s/ZVAD (39.9% ± 8.7, $p<0.01$), and Nec-i/ZVAD (37.8% ± 10.6, $p<0.05$) (**Figure 2B**). In the 48-hour timepoint, significant improvements in vitality are observed between the control (18.7% ± 3.7) and Nec-i/ZVAD (36.6% ± 6.6, $p<0.05$) groups, between the control and Nec1/ZVAD groups (41.6% ± 0.8, $p<0.01$), between Nec-1 (20.8% ± 0.8) and Nec1/ZVAD ($p<0.01$), between Nec-i (12.4% ± 1) and Nec1/ZVAD ($p<0.0001$), and between Nec-i and Nec-i/ZVAD ($p<0.001$) (**Figure 3B)**.

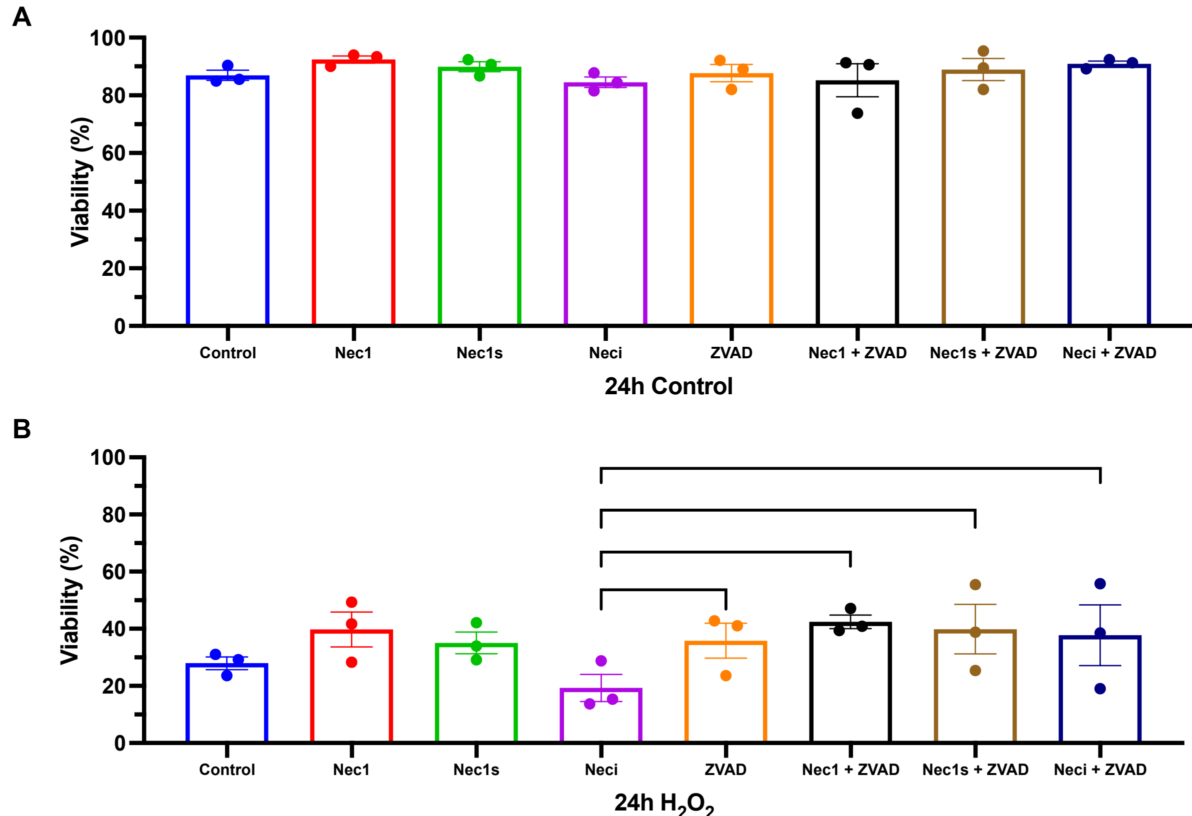


Figure 2. Membrane integrity staining with trypan blue to examine cell viability in MIN6 cells, following 24 hours incubation in apoptosis and necroptosis inhibitors, ± 2 hours incubation with H_2O_2 (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=3). Staining indicates improved vitality following 24-hours incubation in inhibitors and following cell death initiation, between the necrostatin inactive control, and all ZVAD-containing groups. **A)** 24 hours incubation with inhibitors. **B)** 24 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2 (*$p<0.05$, **$p<0.01$).

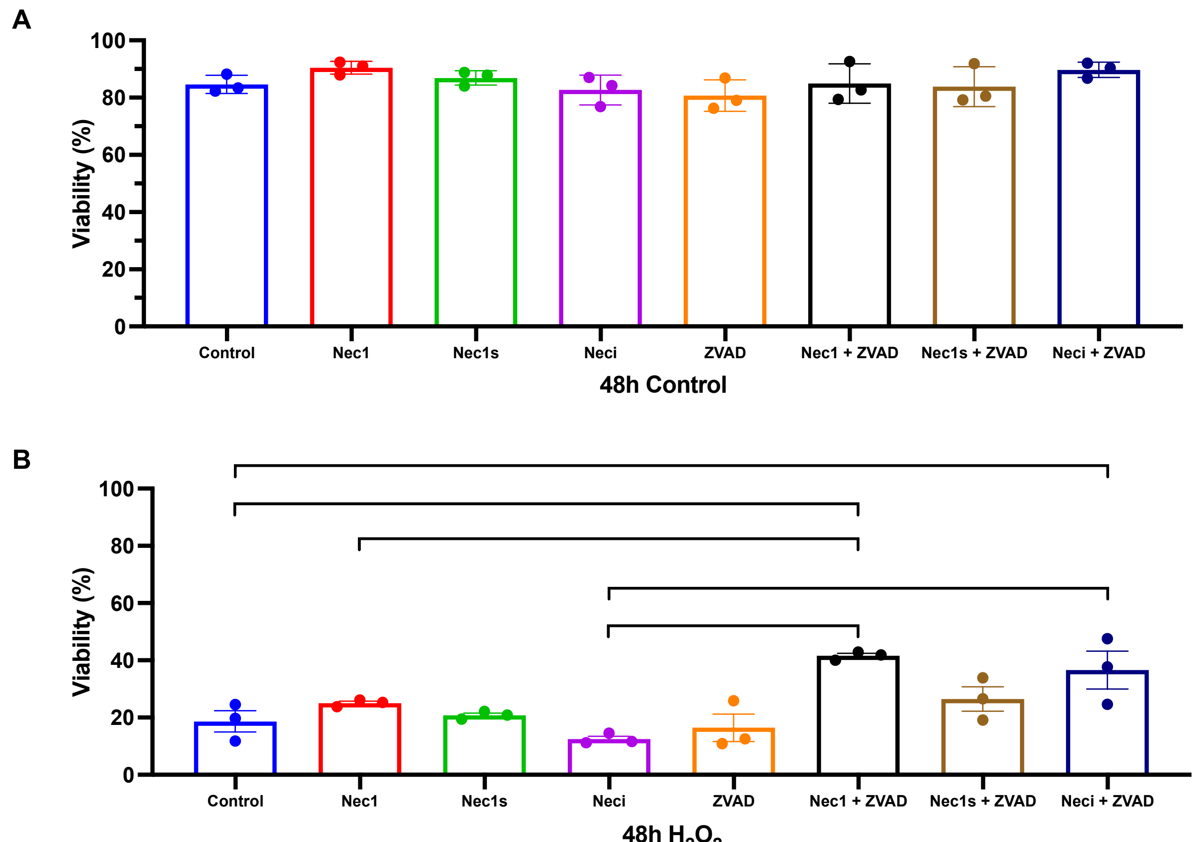


Figure 3. Membrane integrity staining with trypan blue to examine cell vitality in MIN6 cells, following 48-hours incubation in apoptosis and necroptosis inhibitors, ± 2 hours incubation with H_2O_2 (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=3). Staining indicates improved vitality following 48-hours incubation in inhibitors and following cell death initiation particularly when apoptosis and necroptosis inhibitors are applied in conjunction; strength of association is strongest when examining differences between control and Nec1/ZVAD groups, and Nec-i and Nec1/ZVAD groups. **A)** 48 hours incubation with inhibitors. **B)** 48 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Oxygen consumption rate, assessed as a proxy for cellular function, can be measured. Similar to viability measurements, OCR rates, measured as fold change from control, remained consistent at the 24- and 48-hour timepoints prior to H_2O_2 administration (**Figure 4A, Figure 5A**). Following H_2O_2 administration at the 24-hour timepoint, significant differences were observed between control and Nec1s/ZVAD (3.51 ± 1.61, $p<0.001$) and between Nec1s (1.78 ± 0.34) and Nec1s/ZVAD ($p<0.05$), suggesting improved OCR rates following apoptosis and/or necroptosis inhibition (**Figure 4B**). OCR rates did not differ between conditions at the 48-hour timepoint following H_2O_2 administration (**Figure 5B**). However, it can be observed that following addition of apoptosis and necroptosis inhibitors alone or in conjunction with one another, mean OCR rates nearly doubled across all groups as compared with the control group, potentially suggesting that increasing replicates would lead to more illuminating results. OCR was also assessed at a lower H_2O_2 concentration, in order to observe whether the concentration of a cell death initiator altered OCR rates, however, significant differences were not observed between any of the groups at a 200μM concentration (**Supplemental Figure 2**).

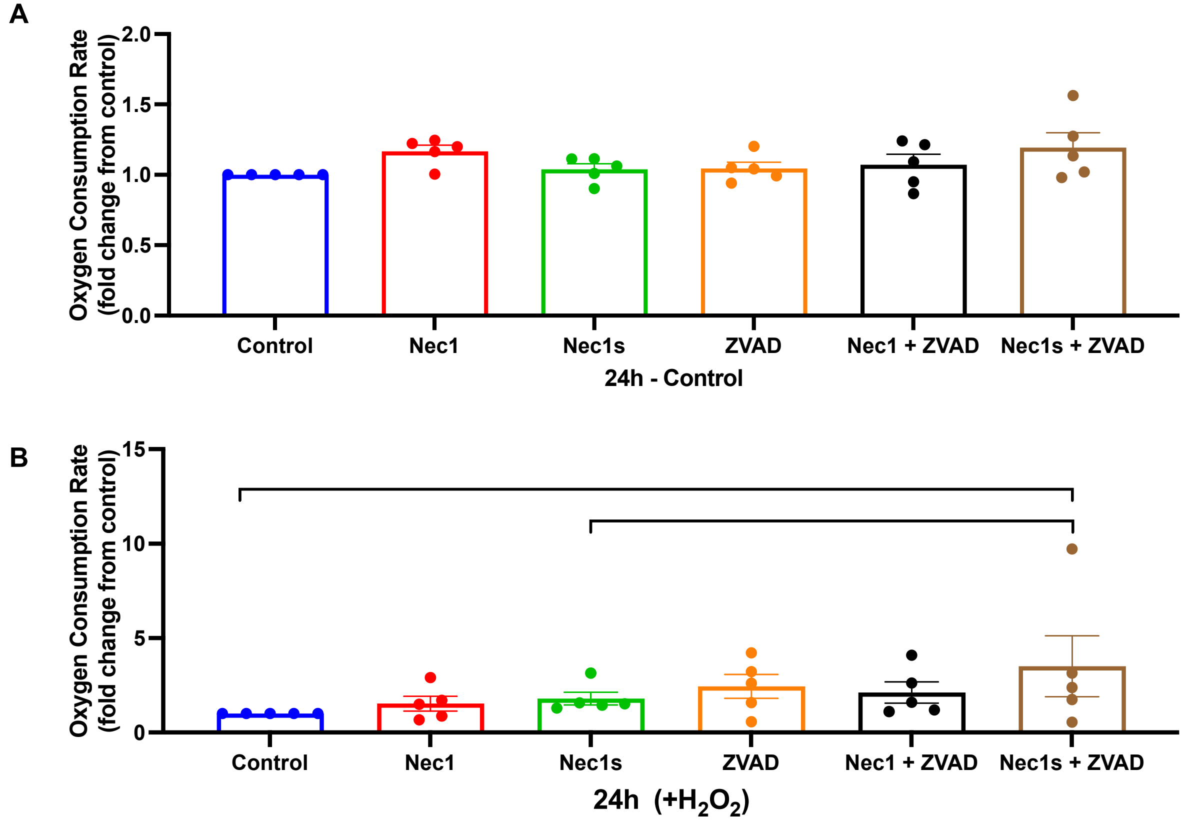


Figure 4. Improved oxygen consumption rate is indicated following 24 hours incubation and initiation of cell death between control and Nec1s/ZVAD groups in MIN6 cells. Data collected following 24-hours incubation in apoptosis and necroptosis inhibitors and ± 2 hours incubation with H_2O_2 (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=5). **A)** 24 hours incubation with inhibitors. **B)** 24 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2. (*p<0.05, ***p<0.001).

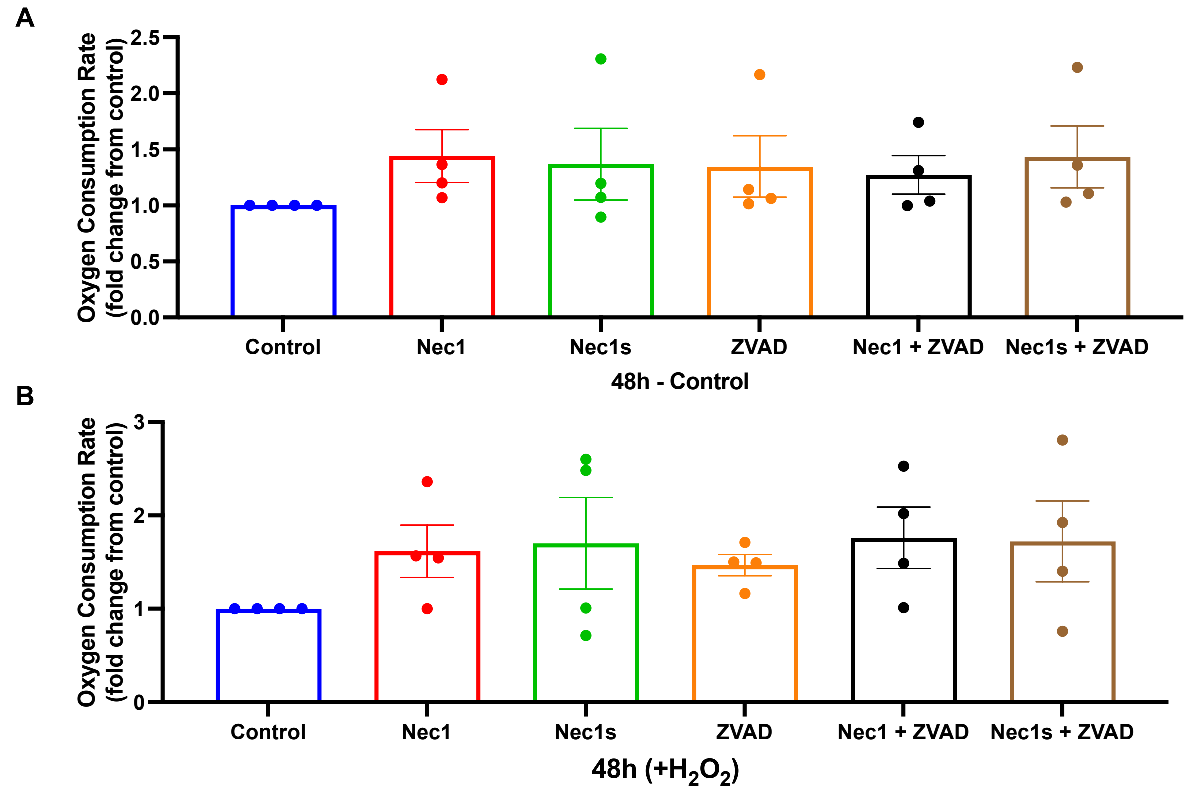


Figure 5. Oxygen consumption rate following 48-hours incubation in inhibitors and following cell death initiation indicates trends towards increasing OCR in MIN6 cells, as compared with control. Data is collected following 48-hours incubation in apoptosis and necroptosis inhibitors and ± 2 hours incubation with H_2O_2. (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=4). **A)** 48-hours incubation with inhibitors. **B)** 48-hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2.

A third metric, GSIS, can also be assessed within MIN6 cells, however, disparate rates of insulin secretion were not observed at the 24- or 48-hour timepoints, with or without cell death initiation (**Supplemental Figure 3**).

Finally, inflammation within MIN6 cells can be assessed within the context of apoptosis and necroptosis inhibition, and initiation of cell death. The inflammatory markers CCL2, CXCL10, ICAM1, IL6, TNF, and TNFAIP3/A20, along with RIPK1 and RIPK3 were examined via PCR for RNA expression following 48 hours incubation in apoptosis and necroptosis inhibitors. When normalized to control levels of expression, significant variability between replicates is observed (**Supplemental Figure 4**).

We can conclude from this set of experiments that apoptosis and necroptosis inhibitors both alone and in combination are able to exhibit significant protective effects on MIN6 cells in the face of cell death initiated by H_2O_2. However, the extent and magnitude of these effects is not fully evident. Moreover, understanding of the conservation of the necroptosis pathway within human islets is yet to be elucidated.

2.2.6 *Characterization of inhibition of apoptosis and necroptosis in human islets*

Given the responses to inhibition in MIN6 cells, it is prudent to examine responses to necroptosis and apoptosis inhibition in a more clinically relevant model. Apoptosis and necroptosis inhibitors can be co-cultured with human islets *in vitro* in order to understand their effect on cell function following a stress.

A GSIS assay was performed following 24 and 48-hours incubation in apoptosis and necroptosis inhibitors and following cell death initiation via H_2O_2 administration. Following 24 hours culture in inhibitors, GSIS is equivalent between all groups, regardless of H_2O_2 administration (**Figure 6A, 6C**). GSIS index, measuring the ratio of high glucose to low glucose secretion, is also equivalent in all groups (**Figure 6B, 6D**). Comparatively, a significant increase in glucose stimulation was observed between the control high glucose condition (2.19% ± 0.53) and the Nec1 high glucose condition (4.47% ± 0.28, $p<0.05$), at the 48-hour timepoint in the absence of H_2O_2 (**Figure 7A**). All GSIS indexes (**Figure 7B, 7D**), and GSIS following H_2O_2 administration (**Figure 7C**) resulted in equivalent rates when compared between groups.

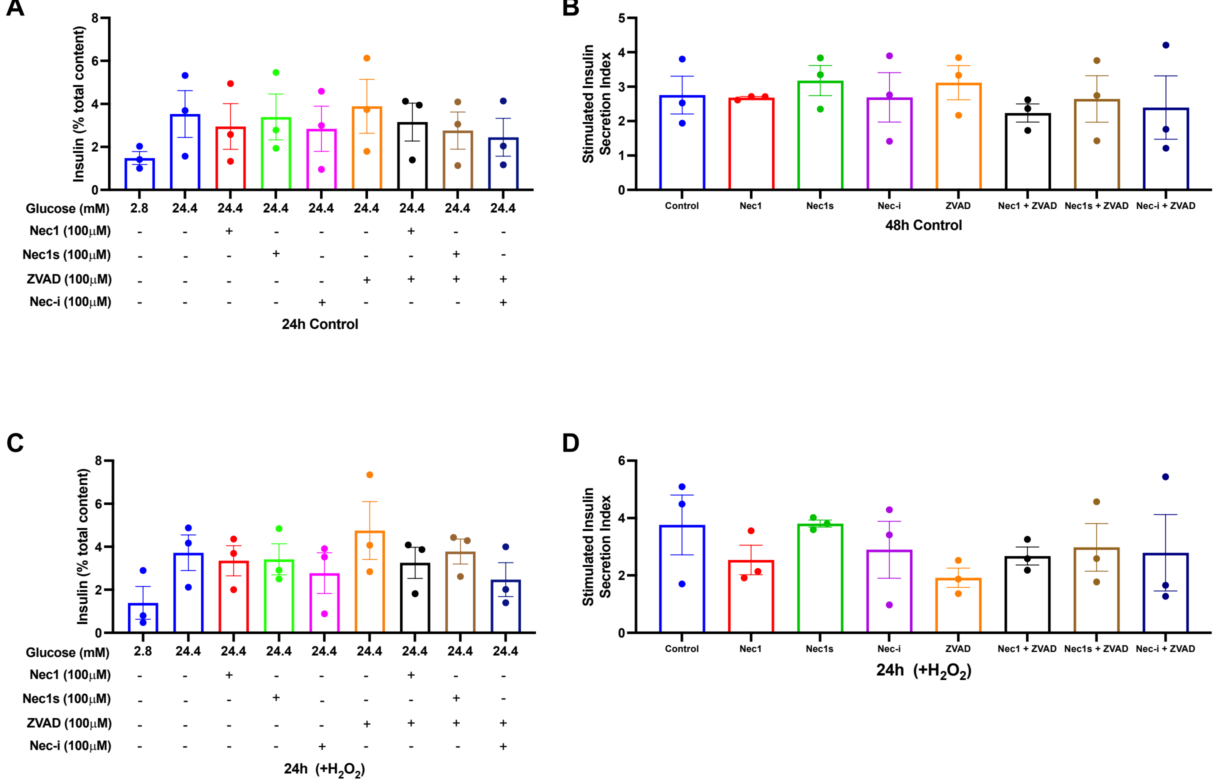


Figure 6. GSIS assay in human islets following 24-hours incubation in apoptosis and necroptosis inhibitors and ± 2 hours incubation with H_2O_2 (one-way ANOVA, Dunnett's multiple comparison test, mean ± SEM, n=3). Data is normalized to total insulin present. **A)** 48 hours incubation with inhibitors. **B)** Corresponding control STIM indexes. **C)** 48 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2. **D)** Corresponding STIM indexes following 2 hours incubation with H_2O_2.

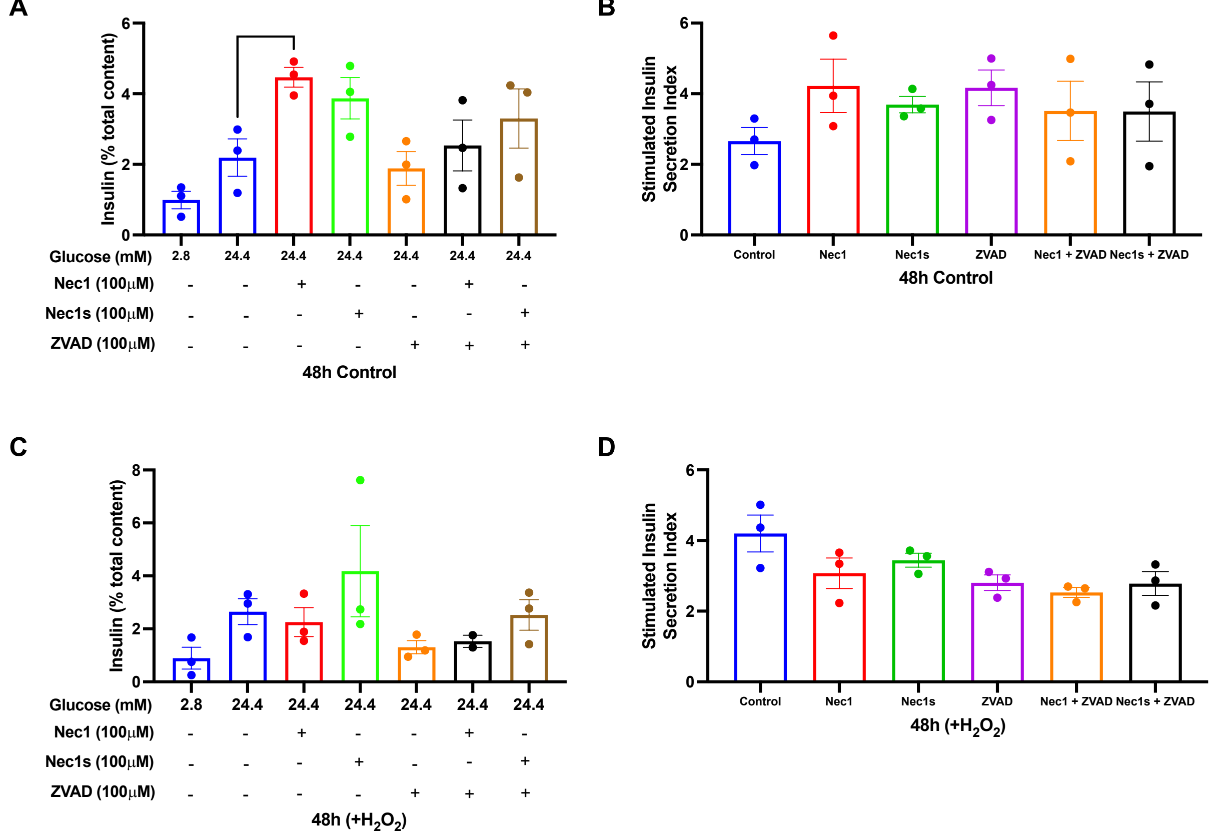
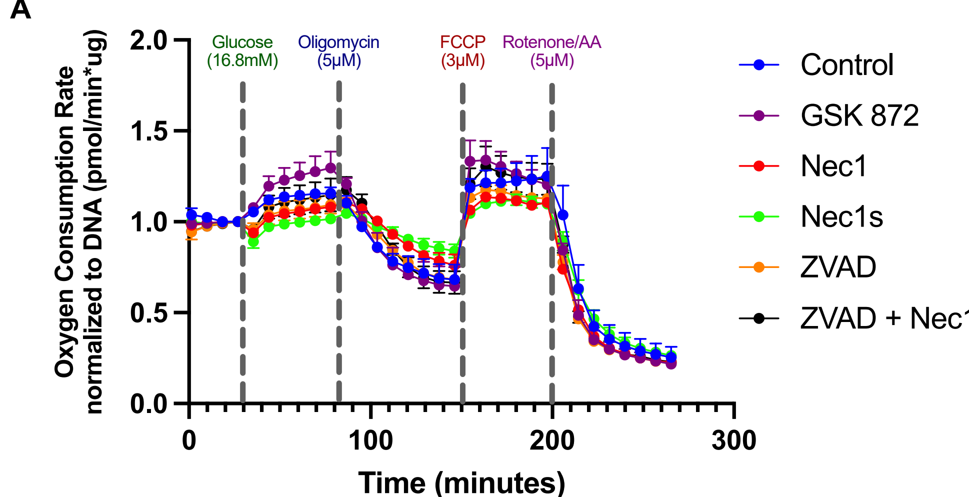


Figure 7. GSIS assay in human islets following 48-hours incubation in apoptosis and necroptosis inhibitors and ± 2 hours incubation with H_2O_2. (one-way ANOVA, Dunnett's multiple comparison test, mean ± SEM, n=3, *p<0.05). Data is normalized to total insulin present. **A)** 48 hours incubation with inhibitors. **B)** Corresponding control STIM indexes. **C)** 48 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2. **D)** Corresponding STIM indexes following 2 hours incubation with H_2O_2.

Additionally, measurement of oxygen consumption within the human islets can be performed using a Seahorse XFe24 extracellular flux analyzer (**Figure 8A**). Five different experimental conditions were tested in this assay – GSK'872, a RIPK3 inhibitor, Nec1, Nec1s, ZVAD, and Nec1/ZVAD. When normalized to total DNA present within the islets, a significant increase is seen between the control and the Nec1/ZVAD conditions (37.48 pmol/min*µg ± 8.07 vs 67.95 pmol/min*µg ± 4.86, $p<0.05$) (**Figure 8B**), indicating an increase in oxygen consumption. Other parameters measured by this assay are presented in **Supplemental Figure 5**.

Furthermore, a similar assay can be performed with the use of 5µM thapsigargin to initiate cell death in the place of H_2O_2 (**Supplemental Figure 6**). However, this did not yield significant results, likely due to the low concentration of the thapsigargin. Moving forward, a dose response curve can be constructed in order to better understand the effect of ER stress on islets.



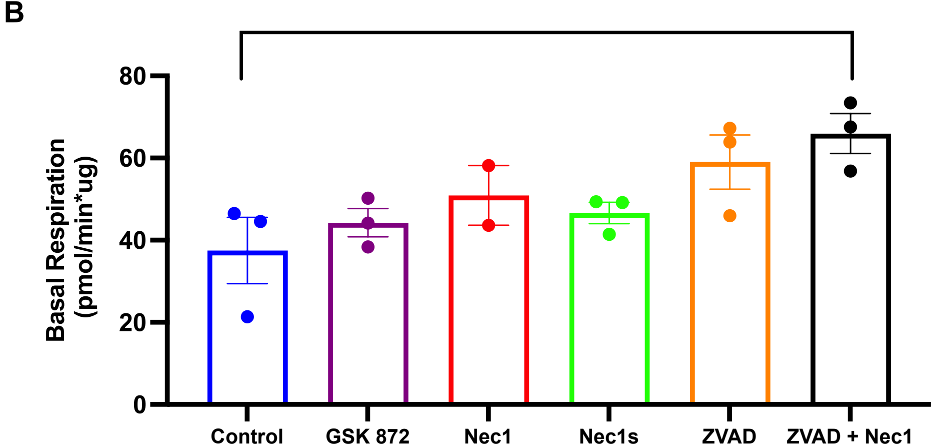


Figure 8. Cellular respiration of human islets measured 72 hours after administration of apoptosis and necroptosis inhibition and ± 2 hours incubation with H_2O_2. A) Oxygen consumption rate measured at basal conditions, and in response to mitochondrial inhibitors. B) Basal respiration, normalized to DNA, indicated significant improvement between control and Nec-1/ZVAD conditions (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, $*p<0.05$).

2.2.7 *Marginal mass transplantation in diabetic mice*

Given the promising *in vitro* responses to targeting regulated cell death, the assessment of the function of apoptosis and necroptosis inhibition was then examined in an *in vivo* model. Mouse allogenic islet transplants of 500 BALB/c islets delivered into diabetic C57BL/6 mice indicated RNA upregulation of RIPK1 and RIPK3 following graft retrieval at experimental endpoint of 7 days, and subsequent RNA analysis (**Supplemental Figure 7**), indicating a potential translational therapeutic target.

Consequently, a total of 41 mice were transplanted with marginal masses of human islets (500 islet equivalents per recipient); 9 islet donors are represented within the data. Islets were pre-treated for 24 hours with apoptosis and necroptosis inhibitors prior to transplant. Metabolic follow-up on transplanted mice was performed to assess glycemic status. Glucose levels decreased within 3-14 days following transplant, and largely remained consistent until grafts were removed 60 days post-transplant (**Figure 9A**). Significant differences were observed in non-fasting blood glucose profiles between experimental groups (**Figure 9B**). Control, Nec-i and Nec1s groups were comparable and not significant, whereas the Nec1 ($p<0.05$), ZVAD ($p<0.01$), Nec1s/ZVAD groups ($p<0.05$) had improved glycemic control as compared with the control group. Intraperitoneal glucose tolerance testing was performed 30 days following transplant, and as indicated by AUC, improved glucose clearance was observed in the Nec1 (1681 ± 321.8, $p<0.05$) and ZVAD (1468 ± 203, $p<0.05$) groups, as compared with control (3287 ± 208.5) (**Figure 10**). Euglycemic mice in all cohorts-maintained glucose homeostasis until islet-bearing grafts were removed at day 60, indicating graft-dependent function.

Furthermore, acute graft studies were also performed to observe inflammation and insulin secretory capacity in the short-term period following transplant. Islets were cultured for 24 hours

in inhibitors, and marginal mass transplants into the kidney capsule of diabetic mice were performed. At 7 days following transplant, the graft-bearing kidney was nephrectomised, and the graft was excised and homogenized. PCR analysis of day 0 islets alone (**Supplemental Figure 8**), and day 7 grafts (**Supplemental Figure 9**) was performed to examine RNA expression levels in IL6, IL1b, RIPK1, RIPK3, TNF, PC, PCSK1, PCSK2, Bax, and BCL2. There is significant heterogeneity between replicates, which likely can be attributed to islet donor heterogeneity, and leading to lack of statistically significant results. However, it can be observed that following transplant, RIPK1 expression levels trend lower in the Nec1, Nec1s, and Nec1/ZVAD groups as compared with control, and RIPK3 levels trend lower in the Nec1 and Nec1/ZVAD groups as compared with control. Furthermore, BCL2, an anti-apoptotic protein, is lowest in the ZVAD-containing groups, and Bax, a pro-apoptotic gene is highest in the Nec1 and Nec1s groups. Additional replicates are likely required in order to better understand the relationship between RNA expression and islet graft function.

In sum, improvement of glycemia and glucose clearance are seen following pre-treatment of both apoptosis and necroptosis inhibitions alone, and in conjunction. These results are correlated to MIN6 and islet survival and function *in vitro*, as conservation of the necroptosis pathway between MIN6 cells and human islets, and subsequent improvement of *in vivo* graft function following transplant was observed.

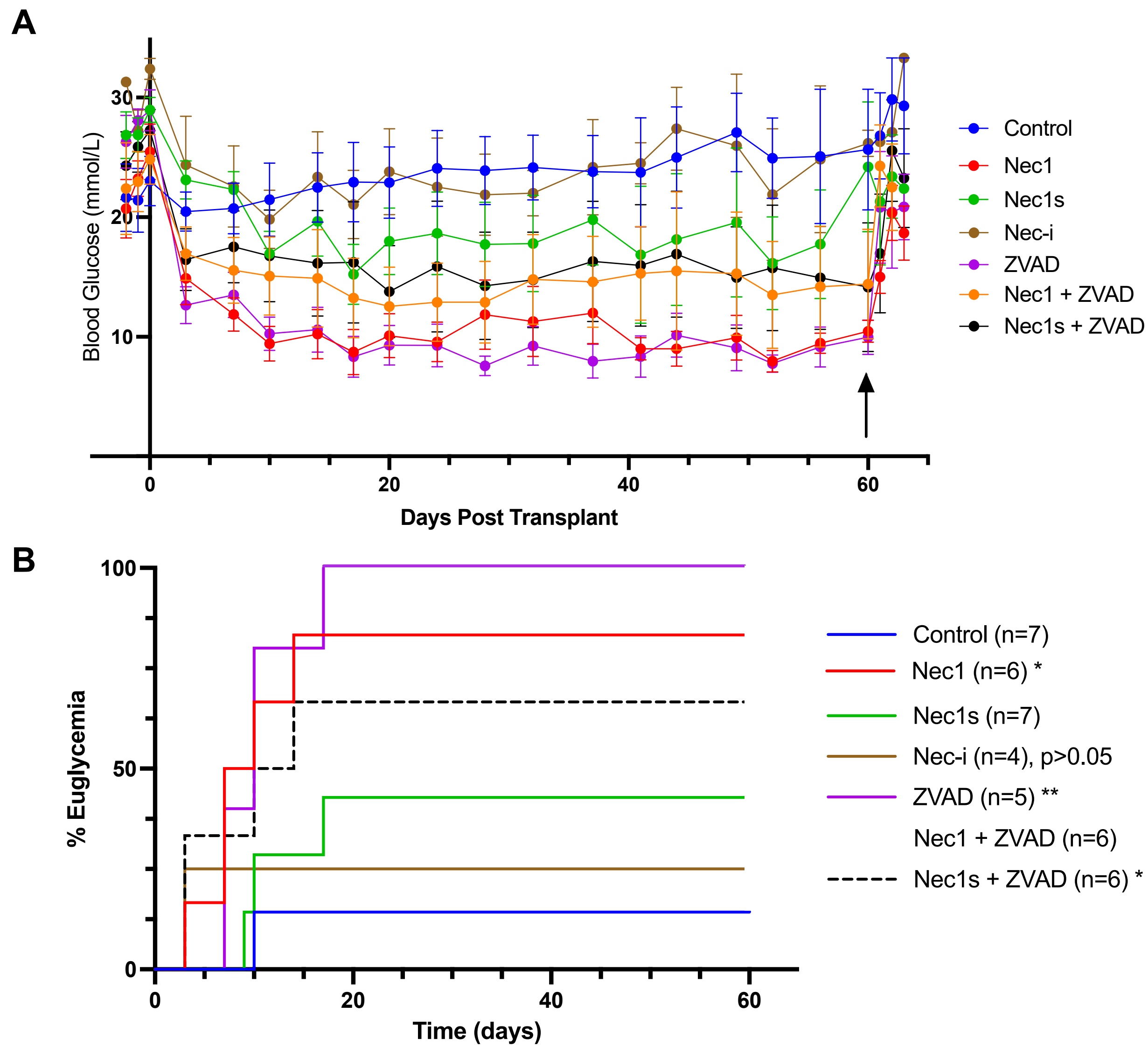


Figure 9. 24 hours of necroptosis and apoptosis inhibition in human islets, followed by marginal mass (500 IEQ) transplantation of islets under the kidney capsule of diabetic mice. **A)** Non-fasting blood glucose measurements of mice recipients' post-transplant. Arrow (60 days) indicates graft retrieval. **B)** Percent euglycemia, indicating reversal of diabetes rate, in each group at 60 days post-transplant (log-rank test, mean ± SEM, *p<0.01, **p<0.01).

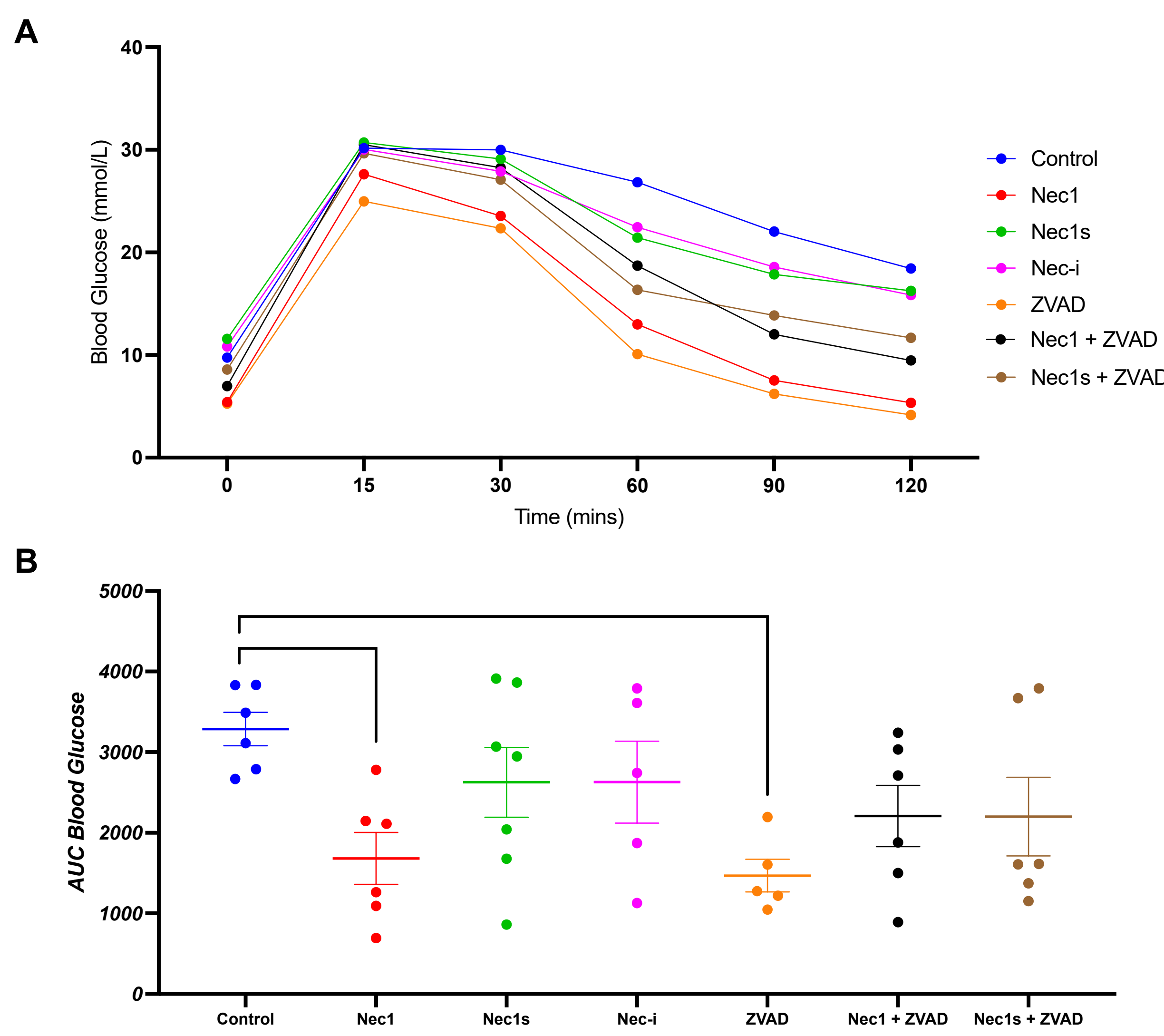


Figure 10. 24 hours of necroptosis and apoptosis inhibition in human islets, followed by marginal mass (500 IEQ) transplantation of islets under the kidney capsule of diabetic mice. **A)** Blood glucose profile during 30-day IPGTT of control and experimental recipients. Mice were administered 3mg/kg 50% intraperitoneal dextrose and blood glucose was measured at t = 0, 15, 30, 60, 90, and 120 minutes. **B)** Mean AUC blood glucose between control and experimental recipients following IPGTT (unpaired t-test, mean ± SEM, *$p<0.05$).

2.4 Discussion

In its current capacity, islet transplantation is limited to those with brittle diabetes, and when conventional insulin therapy has failed. Broadening the scope of islet transplantation would require significant improvements in engraftment efficacy and reduction of cell death, particularly in the acute post-transplant period. In this clinically relevant study, we aimed to determine whether necroptosis and apoptosis inhibition will improve islet survival and function. We demonstrated that culture of MIN6 cells and human islets in necroptosis and apoptosis inhibitors improves cell vitality, oxygen consumption rate, and glucose stimulated insulin secretion. Furthermore, we demonstrated that culture of human islets in cell death inhibitors prior to transplantation under the kidney capsule of diabetic mice results in improved euglycemic rates and improved AUC following IPGTT.

The impetus for this study was determined through observation of the upregulation of RIPK1 and RIPK3 following transplant of islets in a mouse allograft model. As the function of RIPK1 and RIPK3 are well understood in cell death and in necroptosis specifically,[202] this suggested an increase in necroptotic pathways following transplant and indicated a potential avenue for targeting and subsequently reducing cell death, particularly in the acute post-transplant period. Confirmation of the expression of RIPK1 via knockdown in MIN6 cells was performed by siRNA and subsequent western blotting. Observation of reduced expression confirmed the feasibility of targeting RIPK1 in experimental assays, and it was also seen that following vitality and GSIS assays, cell function was preserved in the face of cell stress factors. It should be noted that disparate rates of uptake of siRNA within MIN6 cells across wells may have affected results, however, consistencies were seen when data was analyzed in triplicate.

When considering the therapeutic context, pharmacological inhibition is a more feasible approach, due to a quicker rate of uptake, lower cost, and reduced off-target effects. Therefore,

once altered phenotype following application of siRIPK1 had been established, further experimentation was conducted using pharmacological inhibitors of apoptosis (ZVAD) and necroptosis (Necrostatin-1, Necrostatin-1s, GSK'872). RIPK1 is involved in both the apoptosis and necrosis pathways, however, necroptosis only occurs in the absence of caspase-8, and consequently, application of a pan-caspase inhibitor will prevent RIPK1 dependent apoptosis and shunt cell death activity towards other forms of cell death. This complex and multifactorial role of RIPK1 has also led to research suggesting its involvement as an inhibitor of RIPK3 mediated necroptosis as well. Within a mouse fibroblast cell line (L929) it was observed that RIPK1 knockdown led to accelerated necroptosis and may act as a dominant negative inhibitor of RIPK3 activation,[205] although these effects were not observed within this study.

Furthermore, downstream effects of RIPK1 activation, most frequently initiated by the TNF signaling cascade, include IKK/NF-kB activation, and the interplay between the two results in a delicate maintenance of homeostasis within the cell[206]. NF-kB functions to regulate pro-inflammatory and pro-survival genes, and in different contexts, can serve to assist or oppose RIPK1. In one pathway, activation of this process leads to transcriptional upregulation of genes such as NLRP3 and pro-IL-1B, resulting in inflammasome activation and eventual cell death. This process has also been linked with inflammation and insulin resistance associated with T2DM[207], indicating that a nuanced approach must be undertaken when considering RIPK1 inhibition. In a similar manner, NF-kB has also been linked with RIPK3 associated processes. ER stress, induced by thapsigargin, activates RIPK3.

Nec1 functions as allosteric inhibitors of RIPK1, binding with RIPK1 at the back pocket of its ATP binding site and stabilizing its inactive conformation.[179] Nec1 acts on both RIPK1

and RIPK3, and Nec1s is a highly specific inhibitor for RIPK1 only. While Nec1 inhibits multiple parts of the necrosome, it also interferes with indoleamine 2,3-dioxygenase, an important part of the immune system,[186] potentially leading to other negative systemic effects. Dose responses curves indicated the use 100µM of Nec1 and Nec1s as the most effective concentration, and this is consistent with previous experimentation. Porcine islets cultured in different doses of Nec1 for 7 days resulted in increased recovery, composition of endocrine cells, GLUT2 expression, and insulin secretory capacity only in the 100µM experimental group,[181]

Comparisons were also drawn with necrostatin-inactive (Nec-i), a demethylated variant of necrostatin-1 used as an inactive control to exclude non-specific, off-target effects of necrostatin. Membrane integrity staining in MIN6 cells strongly indicated the feasibility of this approach, as significantly improved cell vitality was observed following 24- and 48-hours treatment with the cell inhibitors under basal and stressed conditions, particularly in the case of the ZVAD, and Nec1/ZVAD groups. Cell stress was initiated via application of H_2O_2 which induces oxidative stress and mitochondrial injury, leading to death. It is a known initiator of necroptosis and apoptosis.[208] Although slightly discrepancies were observed between the control and Nec-i groups, particularly in the 24h incubation assay, leading to differences in statistical significance when compared to the other experimental groups, the two were still considered statistically identical to one another. The differences can potentially be attributed to random error, however, also highlight that divergent rates of cell vitality are not seen until 48 hours of incubation in the inhibitors.

Understanding the effect of apoptosis and necroptosis inhibition on islet function and secretory capacity is of high importance and can be assessed via examination of OCR and GSIS in both MIN6 cells and human islets. Within MIN6 cells, OCR was improved when compared to

control in the Nec1s/ZVAD group in MIN6 cells and improved in the Nec1/ZVAD group in human islets. All other groups indicated no difference in OCR function, indicating that function was not helped or harmed. Cell vitality and OCR results differ from one another, particularly in the 24-hour assay. Cell membrane integrity staining is one of the last observable phenotypic changes within a cell prior to death, and thus, changes in cell membrane integrity occur much further downstream as compared with changes in OCR and GSIS, likely accounting for the differences observed between the two assays.

GSIS in the MIN6 cells showed low levels of stimulation, likely caused by systematic experimental error. As MIN6 cells are cultured in high glucose conditions, and experimented upon in the same dish without dissociation of the adherent cells in between, culture in media lacking glucose is required for a period of time prior to beginning the GSIS assay. PBS washes are performed in between the different glucose conditions to remove any excess insulin present in the cell-culture dish. These washes and media changes may have damaged the cell or the cell's adherence to the dish, influencing experimental results. Additionally, MIN6 cells beyond passage number 40 experience impaired insulin secretion.[209, 210] Care was taken to avoid the use of high passage MIN6 cells, however, insulin secretion may function in a passage-dependent manner, thus, experimental cells could have experienced reduced responses to glucose as they increased in passage number.

MIN6 cells represent a homogenous, glucose responsive, insulin secreting, model of pancreatic β-cells, and permit specific examination of the β-cell without confounding crosstalk with the other cells of the pancreas. However, this ultimately becomes detrimental, as the dissociated cell population behaves differently from native islets, both isolated, and as they function within the pancreas.[211] Thus, examination of GSIS and OCR was subsequently

conducted with human islets. Within the islet, basal respiration was improved in the Nec1+ZVAD group, indicating increased oxygen consumption in order to meet the cellular ATP demand of the cell. Increased metabolic activity within this group may be linked to increased insulin exocytosis. Accordingly, GSIS was improved in the Nec1 group as compared with control in human islets, and all other groups remained statistically similar to the control group. Uptake of glucose via GLUT 2 leads to glucose phosphorylation within the cell, production of ATP, and subsequent insulin secretion. This process is augmented by complex auxiliary metabolic pathways which are not fully established.[212] Krebs cycle activation and the mitochondrial electron transport chain generates some of these signals. Inhibition of the electron transport chain results in reduced insulin secretion in response to glucose.[213] Conversely, increased metabolic activity within human β-cells has also been suggested to indicate chronic glucose stress and accumulation of intermediates in the glycolytic pathway, pentose phosphate pathway, and TCA cycle.[214] Increased cellular glucose, leading to glycolysis and glycolysis byproducts such as ROS, may also lead to necroptosis.[215]

Human islets isolated from a donor are of a heterogenous nature,[216] which stems from differences in donor characteristics such as age, BMI and DCD vs DBD, and differences during islet isolation and handling leading to variability in factors such as purity rates and cold ischemia time. These metrics may also influence the results obtained. It is also possible that given the differences in cellular architecture between MIN6 cells and human islets, increased doses of H_2O_2 are required to adequately stimulate stressed conditions within the human islets. This can be examined via construction of dose response curves with H_2O_2 or other cell death initiators.

Overall, these studies demonstrated conserved or improved *in vitro* functionality following treatment with apoptosis and necroptosis inhibitors, and provided justification for

carrying out *in vivo* experimentation, via the use of a clinically relevant transplant model. Human islets were cultured for 24 hours in necroptosis and apoptosis inhibitors prior to transplantation. Although *in vitro* results suggested improved vitality, OCR and GSIS following 48 hours of culture in inhibitors, and human islets are routinely cultured for 12-72 hours prior to transplantation in practice,[217, 218] culture beyond 20h results in significantly decreased IEQ yield.[219] Islets were transplanted under the kidney capsule of diabetic Rag-1 deficient mice. These mice have small lymphoid organs, and thus do not produce mature T or B cells.[220] Marginal mass transplantation into diabetic Rag-1 deficient mice permits examination of graft function by assessing time to euglycemia, as the islets are not targeted by the natural immune system. Following transplant, shorter time to euglycemia was observed in the Nec1, ZVAD, and Nec1s+ZVAD groups, and euglycemia was maintained until grafts were removed at the end of the study. IPGTT testing revealed lower total rise in blood glucose and quicker return to euglycemia in the Nec1 and ZVAD groups. Taken together, these results suggest that culture in these inhibitors confers significant protection from post-transplant cell death caused by necroptosis and apoptosis.

Acute graft studies to examine proinflammatory cytokines in the acute period following transplant suggested potential reduction of inflammation. A trend of reduction in CXCL10 and IL6 is observed following culture with necroptosis and apoptosis inhibitors and under stressed conditions, suggesting acute reduction in inflammation within the newly transplanted graft. In parallel, inflammatory cytokine analysis with MIN6 cells cultured with cell death inhibitors under basal and stressed conditions, and human islets cultured with cell death inhibitors under basal conditions, hinted towards similar trends but on the whole, results were not illuminating. It

is likely that additional replicates are required to observe statistical significance within this experiment and fully tease out the effect of cell death inhibition on acute inflammation.

The main strength of the study lies in the ability to see strong differentials in post-transplant glycemic levels following solely 24 hours pre-treatment of islets in culture. Apoptosis inhibition provided both via pre-culture of islets and through systemic inhibition following transplant has been trialed numerous times previously,[173-175, 221] however, this is the first study to support the sole use of islet pre-treatment in cell death inhibitors. Moreover, the provision of systemic apoptosis inhibition and subsequent off target effects have the potential to affect cells across the body, preventing necessary cell death and leading to the development of cancers.[222] Tumorigenesis is often associated with significant loss or inactivation of caspases, leading to apoptosis inhibition and rapid and aberrant cancerous cellular growth.[223, 224] Consequently, it is encouraging to observe an improvement in glycemic control via the use of combination therapy, but without the use of systemic inhibition.

Another potential avenue for therapeutic targeting of regulated necrosis is through MLKL. The third and most downstream molecule in the necroptosis pathway, oligomerization of MLKL and subsequent insertion into a cell's membrane leads to the creation of membrane-disrupting pores and eventual cell death. MLKL also functions in interacting with other regulated cell death pathways such as apoptosis, pyroptosis, and autophagy, indirectly regulates gene expression, and binds to certain lipids which may lead to hepatic insulin resistance.[225] These factors may have unpredictable effects on islet physiology, and thus, need to be considered carefully prior to introducing MLKL in the clinical context. However, the non-necroptotic effects of MLKL targeting have the potential to function in a complementary manner to its role in regulated cell death. For example, inhibition of MLKL-driven upregulation of genes such as

ICAM-1 and VCAM-1 in endothelial cells[226] may provide additional protective effects to prevent both acute and chronic graft rejection, as the presence of either gene has been positively correlated with graft rejection.[227] Better understanding the role of associated proteins in the process of regulated cell death could allow for improved ability to target and reduce post-transplant islet loss.

The ability for translation of this project to human patients remains in question. Significant differences exist between mouse and human transplantation, potentially reducing the therapeutic effects. Transplantation into the kidney capsule of diabetic mice results in higher rates of engraftment as compared with transplant into the liver via the portal vein, primarily due to exposure of the engrafted cells to blood within the portal vein which triggers an instant blood mediated inflammatory reaction (IBMIR). This project did not target IBMIR, however, cell death inhibition leading to improved engraftment within the kidney capsule will optimistically lead to a degree of improved engraftment even in the face of IBMIR when islets are transplanted into a more clinically relevant site. Generally, transplant into the kidney capsule of human patients is not feasible, due to lack of anatomical space, potential damage to the kidney in diabetic patient who are already considered high risk for kidney complications, and potential for the immunosuppressive medication to also cause damage to the kidney.[228] Additionally, clustering of islets in a restricted space can lead to the impairment of oxygen and nutrients diffusion, and the subsequent risk of developing nephropathy in T1DM patients. Consequently, engraftment rates between mouse and human transplants into the kidney capsule differ significantly.

Further advancement of this study should involve characterizing responses to necroptosis and apoptosis by studying transplantation in a mouse MHC-mismatch allograft model, which

will allow for a more accurate understanding of graft rejection over time. Islets isolated from RIPK3 knock-out mice (C57BL/6J-*Ripk3*^{m1Btlr}/J) can be used in cell functionality assays, to better understand the function of necroptosis in an *in vitro* model. Subsequently, these islets can be transplanted into diabetic BALB/c mice and monitored for rejection. A strong alloreactive immune response is generated in transplanted islets from C57BL/6 mice into BALB/c mice,[229] offering a valuable clinically relevant model to study islet transplant and glycemic function, and remains unexamined thus far. In a kidney transplant experiment, kidneys from RIPK3-knock out mice transplanted into allogenic recipients exhibited improved function as compared to wild-type kidneys.[230] Although RIPK1 knock-out mice result in death shortly after birth,[231] Cre/loxP site specific recombination can induce knockout of RIPK1 in islets specifically to study necroptosis from a more upstream position. Islets can once again be isolated for *in vivo* and *in vitro* experimentation. Lastly, transplant of islets more clinically relevant sites, such as the portal vein, should be considered. Interestingly, in vitro experimentation with microencapsulated human islets and MIN6 cells, co-cultured with 100μM Nec1 and collagen IV led to increased mitochondrial activity on day 7 in a synergistic manner, and reduced cell loss.[232]

Overall, this study presented a novel strategy for the improvement of graft efficacy in a mouse model. Improvements in engraftment observed following transplant of human islets under the murine kidney capsule indicated the therapeutic potential of apoptosis and necroptosis inhibition of islets prior to transplant. Better understanding of the structure and function of the β-cells allows us to better understand factors that influence β-cell death. In a human patient, inhibition of cell death and increased survival rates can lead to improved rates of success following transplantation. Accordingly, this could lead to a higher rate of diabetes reversal, leading to better quality of life for patients. Patients may only require one donor and one

transplant procedure, resulting in lower surgery-related complications, and overall increased numbers of transplant recipients. In addition, following higher rates of transplantation success, islet cell transplantation could be a therapeutic avenue for a wider range of patients. Currently transplants are limited to individuals with brittle or unstable diabetes, as a last line option, due to the challenges associated with the procedure.

CHAPTER 3 – GENERAL DISCUSSION

3.1 General Discussion

T1DM is chronic disease, resulting from improper autoimmune responses against the β-cells within the Islets of Langerhans of the pancreas. Destruction of these β-cells results in a deficit in insulin production and hyperglycemia. Left untreated, this can result in severe complications, including but not limited to nephropathy, retinopathy, neuropathy, and

cardiovascular disease. Exogenous insulin therapy, the mainstay treatment option, is administered by the patient and work to maintain blood glucose levels within a narrow range. However, it is unable to approximate physiological insulin delivery, although advances in technology and the introduction of paired continuous glucose monitors and insulin pumps bring patients closer to optimal control. Additionally, insulin replacement therapy represents a treatment, rather than a cure.

On the other hand, islet transplantation is a promising option that allows patients to experience insulin responses that closely mirror the function of the native pancreas. It is estimated that 1% of Canadians are living with T1DM, however, islet transplantation can only be offered to a slim minority of these patients due to shortages in the availability of islets and the high burden introduced by the necessary use of lifelong immunosuppressive medication following transplant. Particularly in patients with frequent, severe, and unexpected hypoglycemic episodes, islet transplantation would significantly improve glycemic control and quality of life, and so optimizing the transplant procedure in order to increase the number of patients this cure is available to is of high priority. Significant advancements were made in 2000, when 7 patients were able to achieve insulin independence for a period of one year, following the use of the Edmonton Protocol, however, insulin independence was not sustained over 5 years.

Loss of graft function occurs in both an acute and chronic sense. The overarching goal of this thesis was to better characterize the pathways of cell death that affect islet loss within the immediate-post transplant period. Both apoptosis and regulated necrosis have significant roles in this process. Previous research targeting apoptosis required the provision of systemic inhibition therapy rather than just pretreatment of islets prior to transplant, which is unwise in the long run.

Necroptosis inhibition has thus been unexamined within the context of islet transplantation, providing a novel approach to improving engraftment efficacy.

We examined apoptosis and regulated necrosis in both an *in vitro* and *in vivo* context. We were able to demonstrate conservation of the necroptosis pathway across MIN6 cells and human islets, indicating the feasibility of this approach. Following cell death initiation, it was observed that cell and islet function is preserved or improved following addition of pharmacological apoptosis and regulated necrosis inhibitors, both alone and in conjunction with one another. Marginal mass transplants of human islets treated with these inhibitors for 24 hours, into the kidney capsule of diabetic mice, provided a clinically relevant model from which to study graft function and reversal of diabetes. It was observed that human islets treated with the RIPK1 and RIPK3 inhibitor, Nec1, the pan-caspase inhibitor ZVAD, or the RIPK1 inhibitor Nec1s and ZVAD in conjunction, lead to significantly improved graft function. Additionally, following IPGTT, it was observed that mice with islet grafts treated with Nec1 or with ZVAD had significantly improved glucose clearance. This has not been demonstrated in a mouse model previously.

When considering the broader context of therapeutic avenues to treat T1DM, these results have profound implications. Improvement of engraftment efficiency, particularly in the early post-transplant period, would allow for better long term survival rates of islets, higher rates of insulin independence following transplant, and higher rates of single donor and single procedures success rates, leading to long term insulin independence. Improved graft durability over time would increase the islet availability and allow this procedure to be made available to a wider range of patients, offering a much better approximation of physiological insulin delivery than is currently available to the majority of patients with T1DM. However, better understanding of

post-transplant inflammatory responses, along with study of these pathways in more clinically relevant models, is required before this work can be translated into the clinic.

3.2 Summary

In this thesis, we set out to better understand the role of apoptosis and regulated necrosis in an islet transplant model. Islet transplant remains an exciting therapy, as it can best recapitulate the way in which native islets regulate insulin. However, in the clinical setting, it has been observed that up to 80% of islets will die in the 24 hours following a transplant. Paired with

graft rejection, this leads to reduced rates of insulin independence, and the requirement of multiple transplant procedures and islets isolated from multiple human donors for each transplant. By better understanding cell death in the immediate post-transplant period, we hope to be able to improve graft durability and engraftment rates.

The objective of this study was to characterize the function of the necroptosis pathway, and understand its conservation, across MIN6 cells and human islets. Functional studies in both models indicated that pharmacological necroptosis and apoptosis inhibitors did not alter, or improved cellular function, even when faced with cell stresses. Following transplant of treated islets into the kidney capsule of diabetic mice, it was observed that inhibition of both apoptosis and necroptosis, whether alone or in combination, leads to improved graft function and improved glucose clearance, suggesting potential reduced cell death in the post-transplant period.

Improved understanding of islet physiology and cell death may ultimately lead to improved efficacy of transplant in human patients, resulting in better engraftment rates, lower doses of islets required per transplant, and increased availability of transplant to a broader range of T1DM patients.

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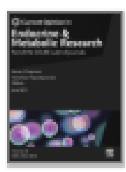
APPENDIX

Current Opinion in Endocrine and Metabolic Research

Volume 24, June 2022, 100354



Reviews

Clinical translation of porcine islets for treating type 1 diabetes

Saloni Aggarwal [1, 2], Andrew R. Pepper [1, 2], Gregory S. Korbutt [1, 2]

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Abstract

Insulin replacement therapy is the mainstay treatment option for type 1 diabetes; however, when this fails to adequately maintain glucose homeostasis, islet transplantation can provide a solution. Shortages and the heterogenicity of human islet donors lead to the desire for an alternative, such as the use of xenografts, particularly porcine islets. Incremental improvements in immunosuppression, islet isolation, and xenograft characterization over the past thirty years have led to the feasibility of this solution. Engraftment of porcine islets can be facilitated through combinations of immunosuppressive reagents, macro or microencapsulation of islet grafts, and the use of genetically modified porcine islets, which are more compatible with the human body, both with respect to the immune system, and to the structure of insulin. Herein, we review the current advancements that may position porcine islet xenotransplantation to become a feasible clinical option for the treatment of type 1 diabetes.

Appendix A-1: First page of publication entitled "Clinical translation of porcine islets for treating type 1 diabetes" published in Current Opinion in Endocrine and Metabolic Research, and authored by S. Aggarwal, A.R Pepper, G.S Korbutt. Parts of this publication are represented within **Chapter 1** of this thesis.

Journal of Immunology and Regenerative Medicine
Volume 16, May 2022, 100058

Augmenting engraftment of beta cell replacement therapies for T1DM

Saloni Aggarwal [a, b], Andrew R. Pepper [a, b], Najwa Al Jahdhami [c]

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Highlights

- β-cell replacement therapy is effective and safe for a subset of T1DM patients with difficult to control hypoglycemia.
- Cellular encapsulation represents a promising strategy to reduce or eliminate the requisite of systemic immunosuppression.
- Extrahepatic transplant sites are currently being explored and may be a suitable environment for alternative **β**-cell sources.
- Co-transplantation with pro-angiogenic cells may increase engraftment efficacy.
- Novel cell death inhibitors provide a unique opportunity to study the mechanisms that govern β-cell graft function.

Appendix A-2: First page of publication entitled "Augmenting engraftment of beta cell replacement therapies for T1DM" published in the Journal of Immunology and Regenerative Medicine, and authored by S. Aggarwal, A.R Pepper, and N. Al Jahdhami. Parts of this publication are represented within **Chapter 1** of this thesis.

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New Results

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Redox sensing by SENP1 augments insulin secretion early after high-fat feeding in mice

Haopeng Lin, Kunimasa Suzuki, Nancy Smith, Xi Li, Lisa Nalbach, Sonia Fuentes, Aliya F Spigelman, Xiaoqing Dai, Austin Bautista, Mourad Ferdaoussi, Saloni Aggarwal, Andrew R Pepper, Leticia P Roma, Emmanuel Ampofo, Wen-hong Li, Patrick E MacDonald

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This article is a preprint and has not been certified by peer review [what does this mean?].

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Abstract

Pancreatic β-cells respond to metabolic stress by upregulating insulin secretion, however underlying mechanisms remain unclear. In β-cells from overweight humans without diabetes, and mice fed high-fat diet (HFD) for as little as 2 days, insulin exocytosis and secretion are enhanced without increased Ca^{2+} influx. β-cell RNA-seq suggests altered metabolic pathways linked to cytosolic redox following HFD. The increased β-cell exocytosis upon HFD is dependent on a shift towards a reduced intracellular redox state, and increased expression of sentrin-specific protease-1 (*Senp1*). Mechanistically, allosteric binding of Zn^{2+} at a site that includes C535 suppresses basal SENP1 activity and unrestrained β-cell exocytosis and increases SENP1 sensitivity to activation by redox signals. Mice with pancreas- or β-cell SENP1 deletion fail to up-regulate exocytosis after 2-day HFD and become rapidly glucose intolerant. This highlights a key role for Zn^{2+}-dependent redox signaling via SENP1 in β-cell functional responses to metabolic stress. (Words: 145)

Appendix A-3: First page of publication entitled "Redox sensing by SENP1 augments insulin secretion early after high-fat feeding in mice," authored by H. Lin, K. Suzuki, N. Smith, X. Li, L. Nalbach, S. Fuentes, A.F. Spigelman, X. Dai, A. Bautista, M. Ferdaoussi, S. Aggarwal, A.R Pepper, L.P. Roma, E. Ampofo, W. Li, and P.E. MacDonald. Saloni Aggarwal provided assistance in running and analyzing OCR and DNA assays for this publication.

Appendix A-4: Supplemental Methods and Figures

Insulin Secretion Assay in MIN6 cells:

MIN6 cells in 6-well plates were incubated for 2h in DMEM without glucose. Following this, a PBS wash was performed. Half the wells were incubated in DMEM containing a low glucose solution (2.8mmol/L), and half the wells were incubated in DMEM containing a high glucose (24.4mmol/L) for 60 minutes at 37°C. The supernatant was then removed and stored at -20°C.

Insulin secretion was standardized to total insulin content. All cells were removed from each well via trypsin-EDTA as described previously and centrifuged at 750rpm for 7 minutes. The supernatant was removed, and 1mL of azol (11.4% v/v glacial acetic acid, 0.25% BSA) was added. Samples were stored at -20°C, sonicated, lyophilized, and resuspended in FSA buffer (10mM monosodium phosphate, 150 mM NaCl, 0.05% BSA).

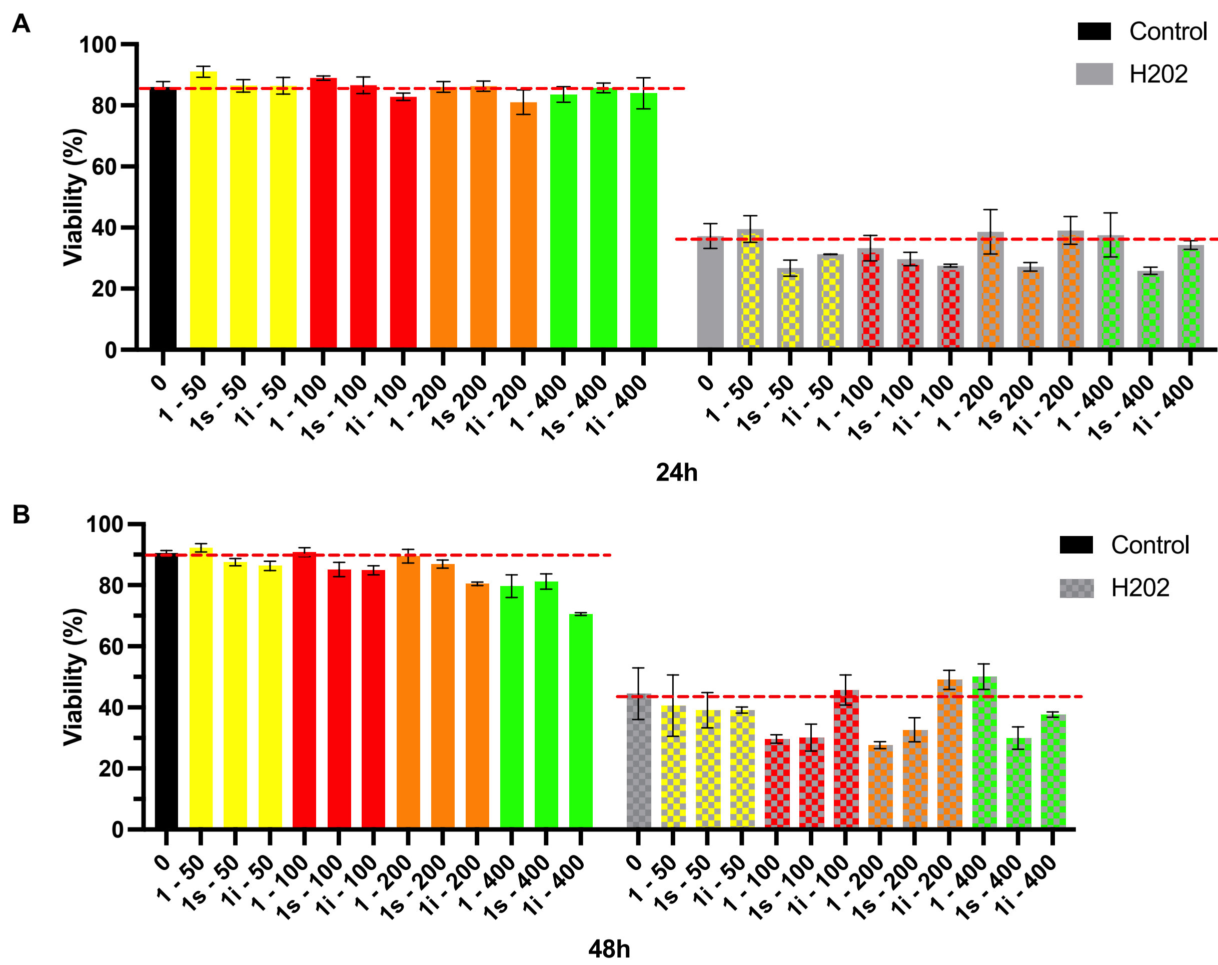
Insulin was measured using a mouse/rat insulin kit as per manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD, USA).

Acute graft studies:

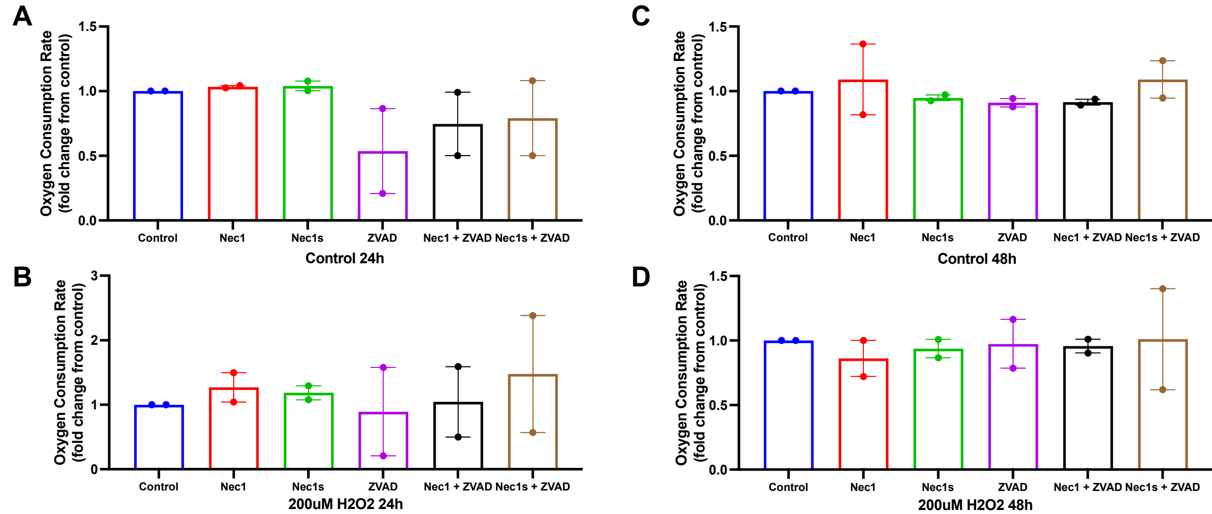
Diabetes was induced and mice were transplanted as per 2.2.3.1 and 2.2.3.2. Non-fasting blood glucose measurements were made at day 1, 3, 5, and 7 following transplantation. At 7 days post-transplantation, all mice underwent nephrectomy of the graft-bearing kidney and subsequent euthanasia. Grafts were homogenized using sterile DNase/RNAse free pestles, resuspended in 1mL TRIzol, and frozen at -80°C.

RNA isolation and RT-PCR:

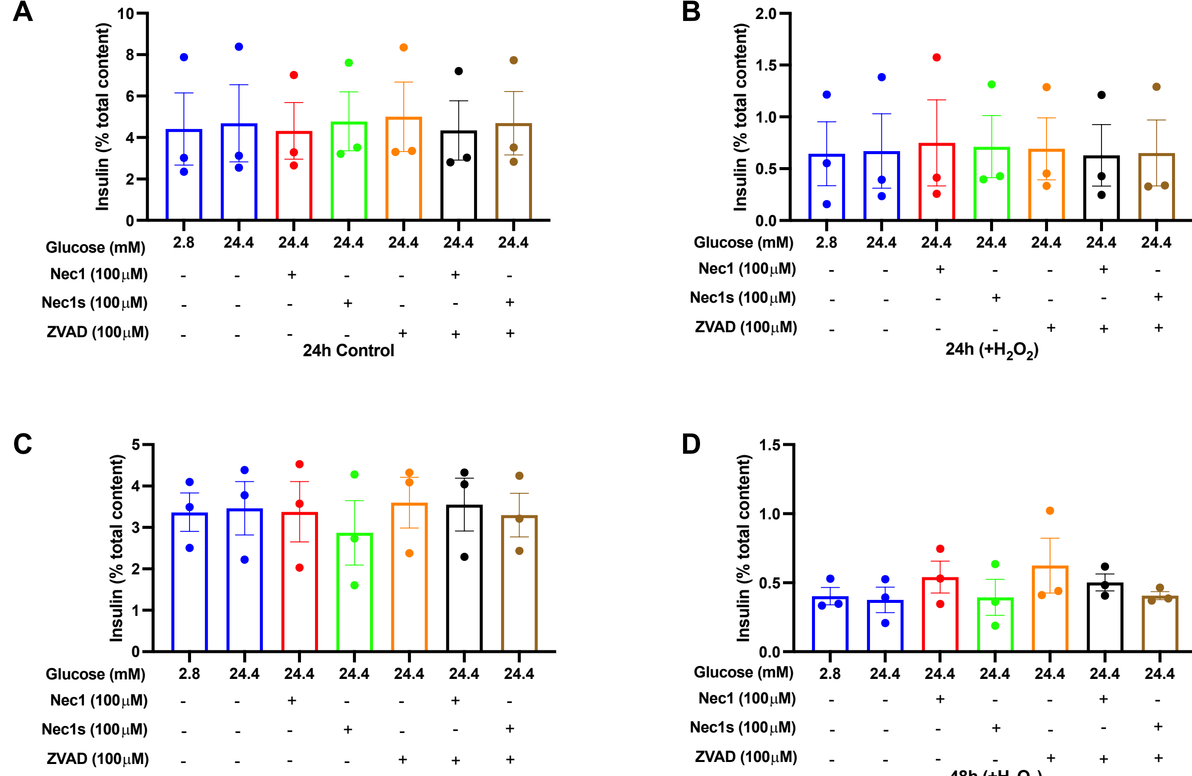
Extraction of RNA was performed using the RNase Easy Micro Kit (Qiagen, Toronto, ON, Canada) as per manufacturer's directions. Briefly, the aqueous phase of each sample was separated via chloroform and transferred to a fresh microcentrifuge tube. RNA was precipitated out via the addition of ethanol. Washes with ethanol, RPE, and RWI in Qiagen columns permitted purification, and samples were diluted with 20uL of RNAase free water. RNA purity and yield was assessed via nanodrop, and 1ug of RNA was reverse transcribed. Resulting cDNA was amplified for 40 cycles, and relative quantification was performed using the ABI PRISM 7900HT. Validated primer sets were as follows: RIPK1 (HS01041869_m1), RIPK3 (HS00179132_m1), TNF (HS00174128_m1), IL6 (HS00174131_m1), IL-**1**β (HS0155410_m1), PC (HS01085875_m1), PCSK1 (HS01026107_m1), PCSK2 (HS00159922_m1), BCL2 (HS00608023_m1), and Bax (HS00180269_m1). Negative controls included non-transplanted mouse kidney, to ensure that primers were not cross-reacting with mouse DNA, and non-transplanted untreated human islets cultured overnight were used as positive controls. Analysis by RQ software was performed using the ΔΔCT method.



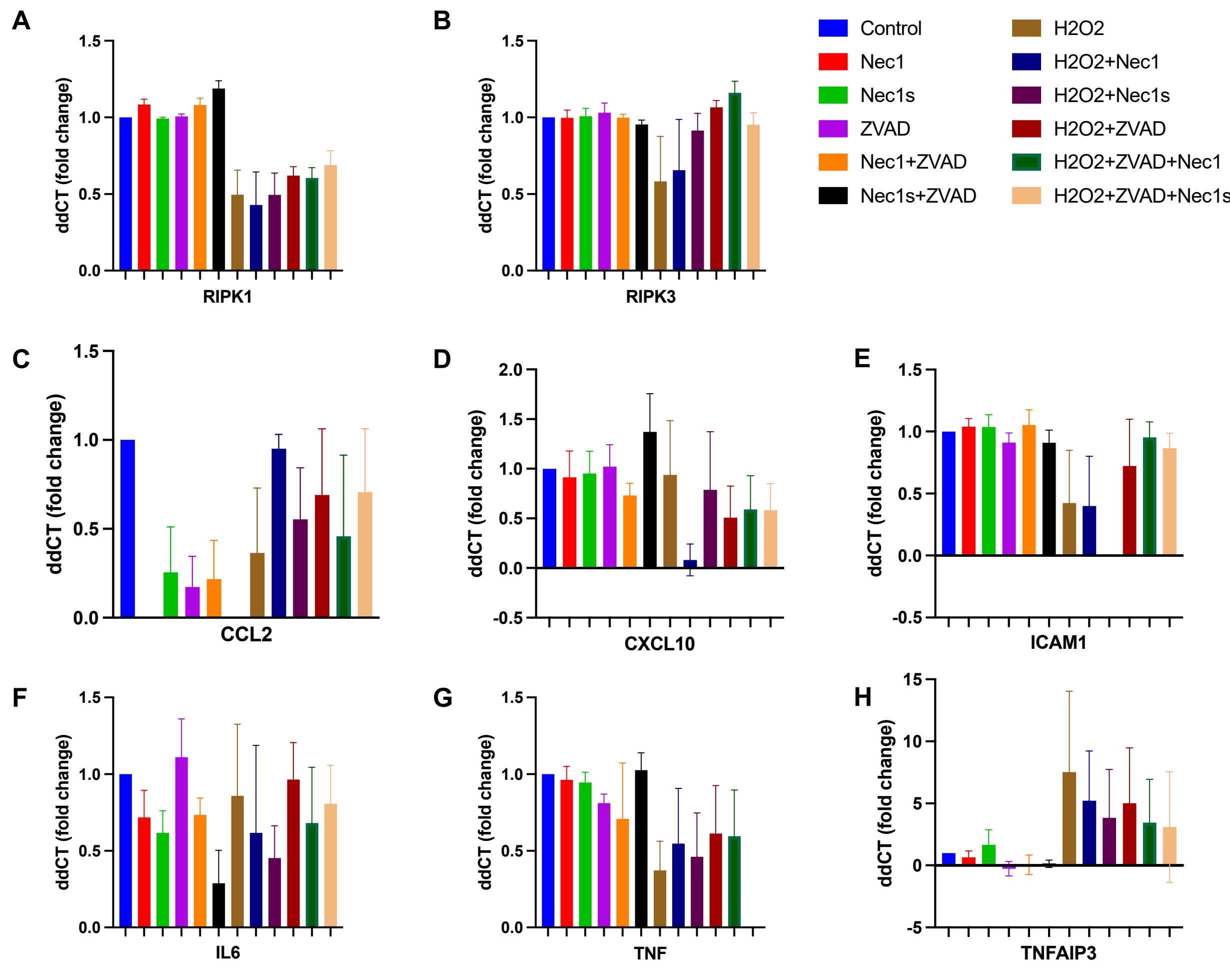
Supplemental Figure 1. A dose response curve assessing various concentrations of necroptosis inhibitors on MIN6 cells under both basal and stressed conditions indicates 100µM as the optimal experimentation concentration following 24 and 48 hours culture. Cells were assessed for vitality via membrane integrity staining with trypan blue (n=3). **A)** Cells were cultured for 24 hours prior to 400µM H_2O_2 incubation in Nec1, Nec1s, and Nec-i. **B)** Cells were cultured for 48 hours prior to 400µM H_2O_2 incubation in Nec1, Nec1s, and Nec-i.



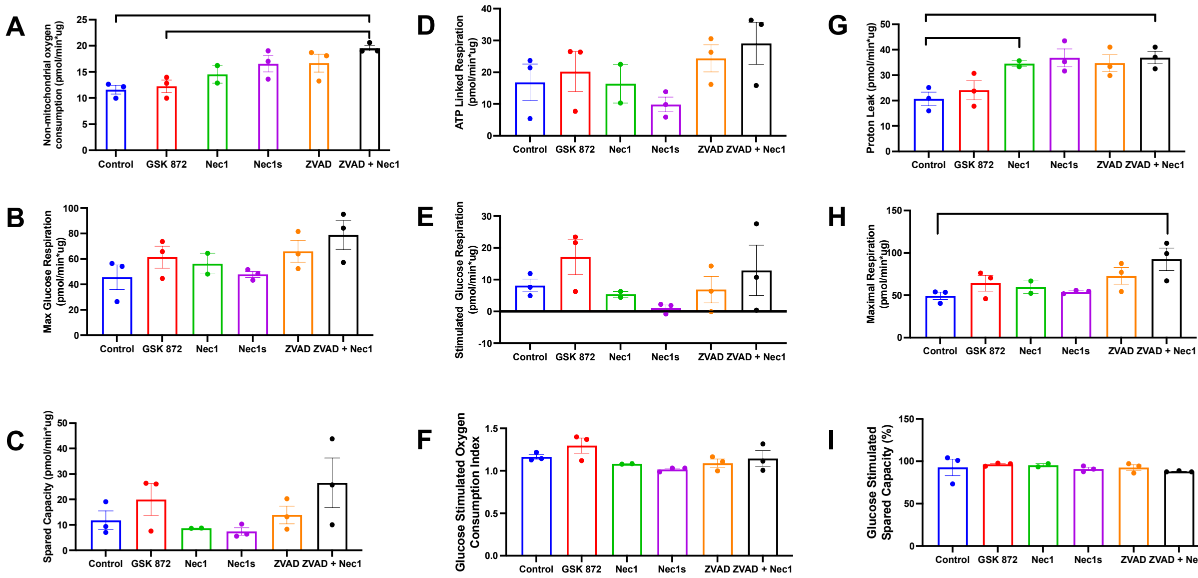
Supplemental Figure 2. OCR assessed following low-dose (200μM) H_2O_2 cell death initiation indicated lack of disparate oxygen consumption between groups, regardless of length of time of cell death inhibitor incubation (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=2). **A)** 24 hours incubation with inhibitors. **B)** 24 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2. **C)** 48 hours incubation with inhibitors. **D)** 48 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2.



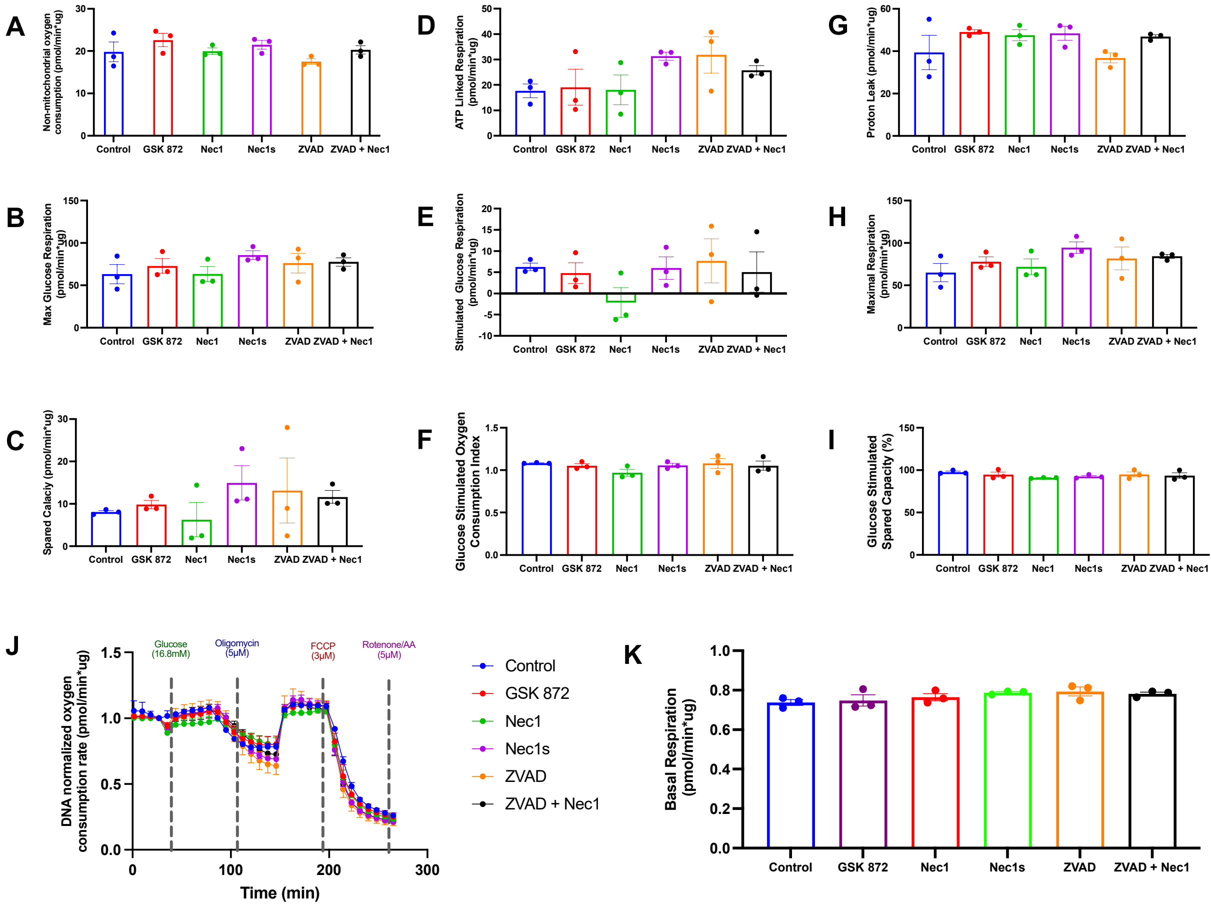
Supplemental Figure 3. Statistically significant GSIS is not observed in MIN6 cells, following 24- and 48-hours incubation in apoptosis and necroptosis inhibitors, ± 2 hours incubation with H_2O_2 (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=3). Insulin secretion is standardized to total insulin content. **A)** 24 hours incubation with inhibitors. **B)** 24 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2. **C)** 48 hours incubation with inhibitors. **D)** 48 hours incubation with inhibitors, followed by 2 hours incubation with H_2O_2.



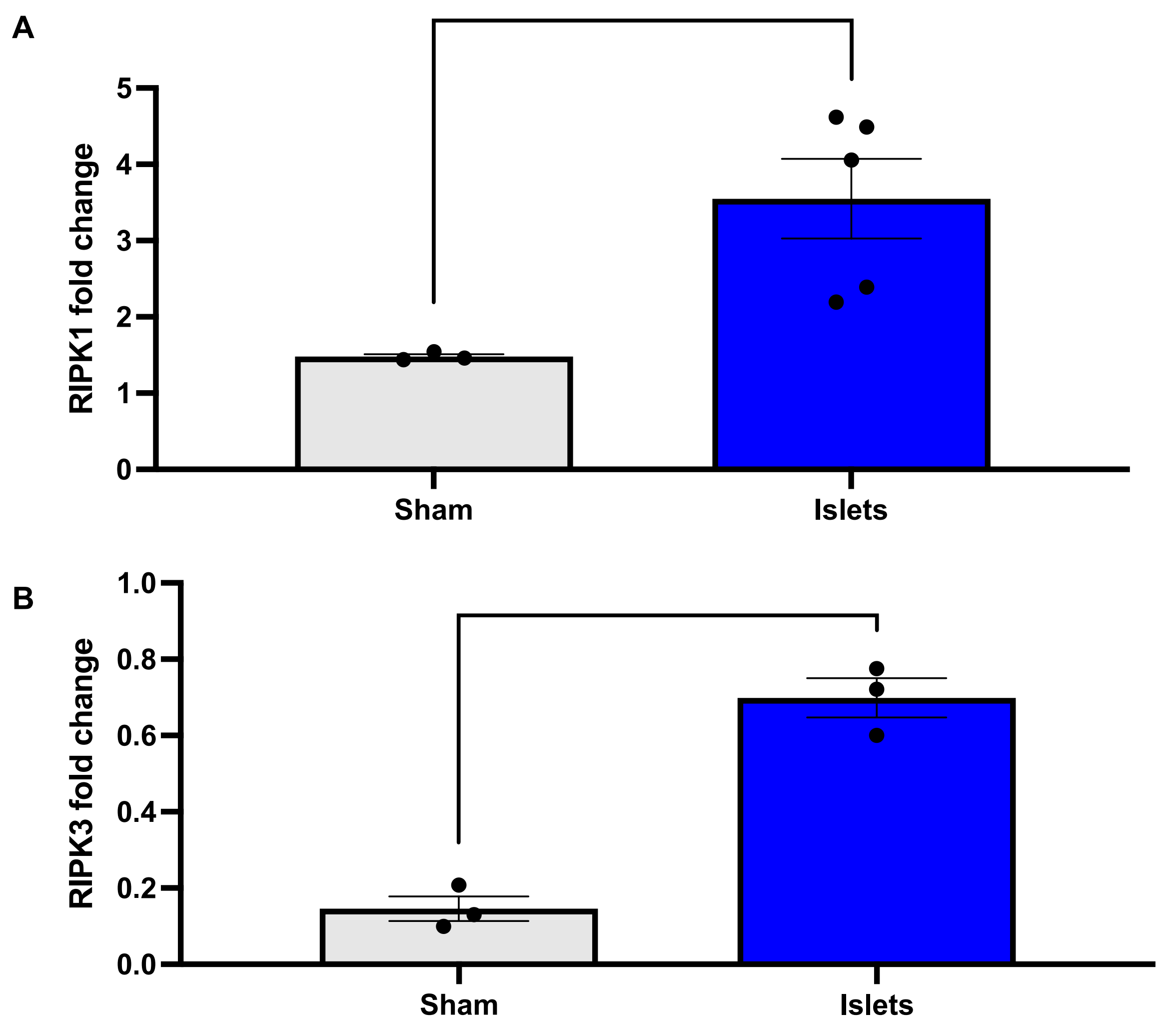
Supplemental Figure 4. RNA levels of inflammatory factors in MIN6 cells, assessed following 48 hours of culture with inhibitors and ± 2 hours incubation with H_2O_2 (mean ± SEM, n=3). Data is normalized to control (islets cultured for 24 hours without inhibitors and without H_2O_2) and presented as fold change. A trend of reduction in CXCL10 and IL6 is observed following culture with necroptosis and apoptosis inhibitors and under stressed conditions, suggesting acute reduction in inflammation within the newly transplanted graft.



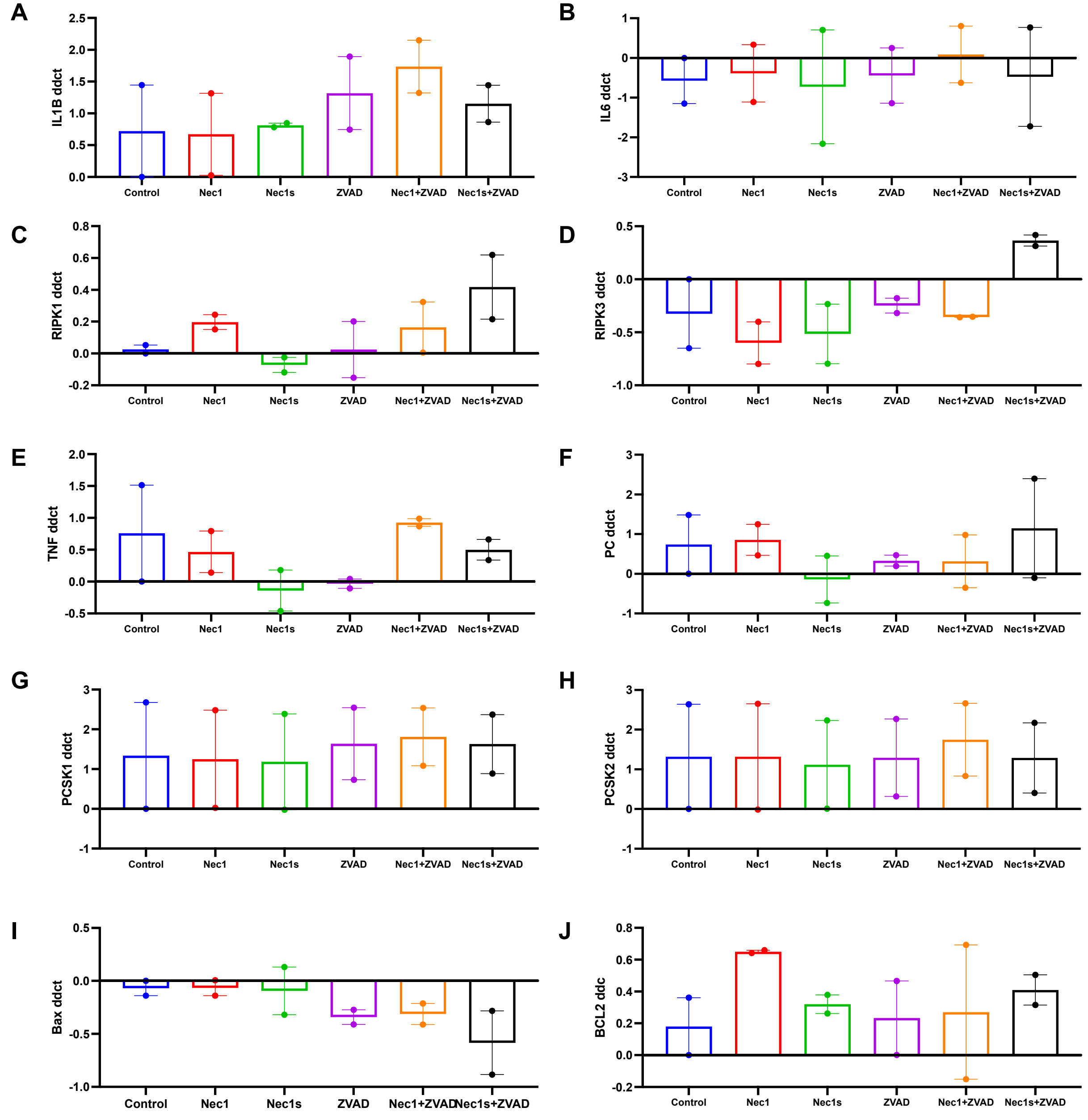
Supplemental Figure 5. Mitochondrial stress test performed via Seahorse XFe24 extracellular flux analyzer. Measurements were performed following 72 hours incubation of human islets with inhibitors, and following 2 hours incubation with H_2O_2 (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM). Data is taken from one islet donor, run in triplicate.



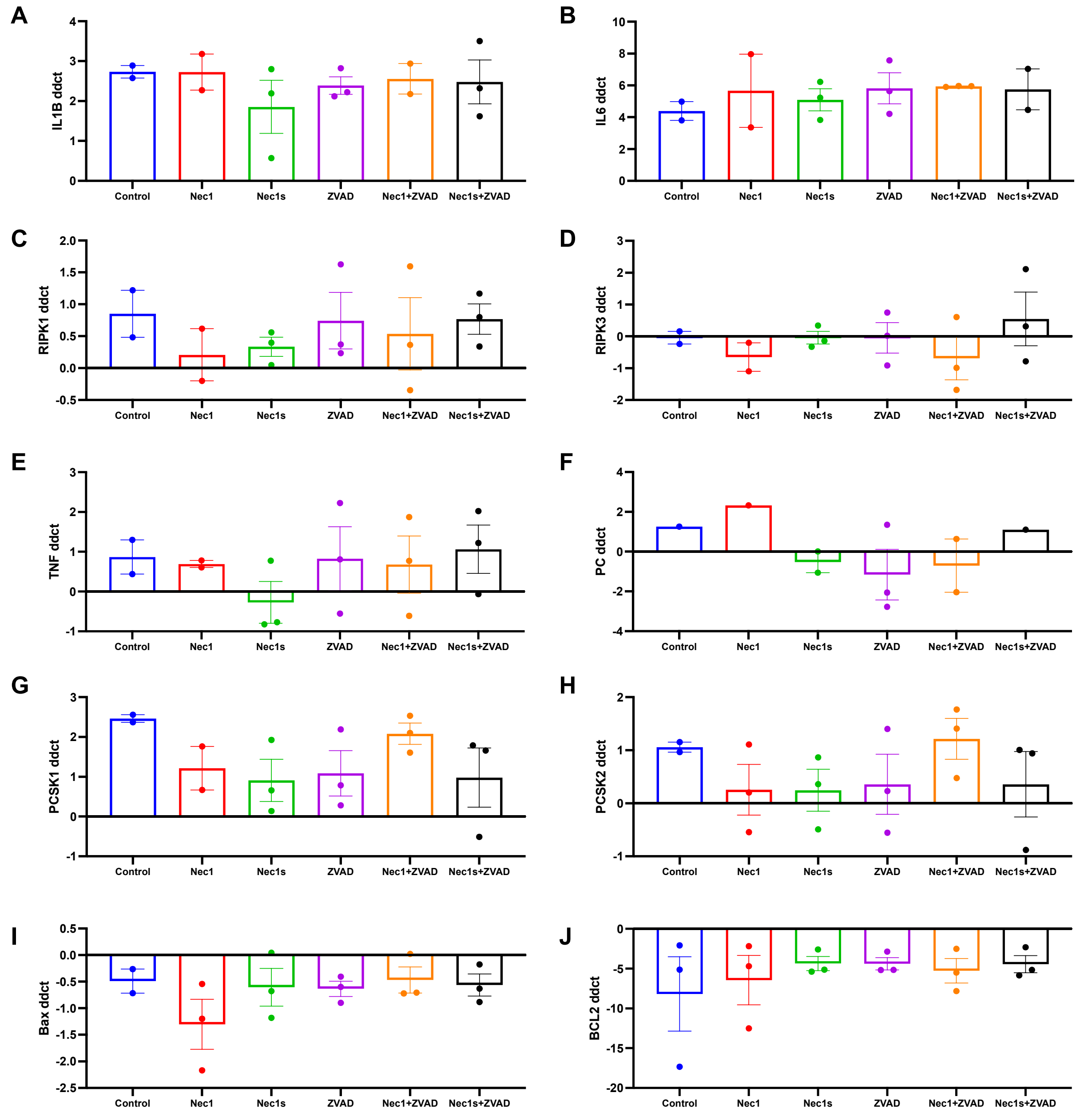
Supplemental Figure 6. Mitochondrial stress test performed via Seahorse XFe24 extracellular flux analyzer. Measurements were performed following 72 hours incubation of human islets with inhibitors, and following 2 hours incubation with thapsigargin (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM). Data is taken from one islet donor, run in triplicate.



Supplemental Figure 7. Upregulation of RIPK1 and RIPK3 is observed following allogenic transplant of 500 BALB/c islets delivered into diabetic C57BL/6 mice under the kidney capsule (unpaired t-test, mean ± SEM, n=3, *p<0.05, ***p<0.001).



Supplemental Figure 8. RNA analysis of human islets following 24 hours culture in inhibitors sampled prior to transplant, and subject to reverse-transcriptase PCR (RT-PCR) for immune regulatory and insulin secretory factors (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=3). Control islets (non-transplanted overnight cultured isolated islets) were used as a baseline.



Supplemental Figure 9. RNA analysis of short-term transplants harvested at day 7, and subject to reverse-transcriptase PCR (RT-PCR) for immune regulatory and insulin secretory factors (2-way ANOVA, Tukey's multiple comparison test, mean ± SEM, n=3). Control islets (non-transplanted overnight cultured isolated islets) were used as a baseline.