THE ROLE OF TOLL-LIKE RECEPTORS IN ISLET ALLOGRAFT REJECTION AND NORMAL BETA-CELL FUNCTION

by

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Abstract

Diabetes mellitus is both a metabolic and inflammatory syndrome, characterized by hyperglycemia in the presence of insufficient beta-cell mass and high levels of circulating proinflammatory cytokines. Determining the mechanism behind this chronic inflammation is important for developing therapeutic targets, as controlling inflammation may prevent the progression of diabetes, development of secondary disease, and failure of islet allografts. Toll-like receptors (TLRs), a family of pattern recognition receptors of the innate immune system, are important for regulating adaptive immunity. The aim of this project was to determine the role of Toll-like receptor signalling in islet transplantation and normal beta-cell function.

The role of TLR signalling in islet transplantation was assessed using a full major histocompatibility complex mismatch murine islet allograft model. TLRs are capable of sensing both exogenous and endogenous ligands. Excessive TLR activation during transplantation may promote allograft rejection. To test this hypothesis, islets from TLR-deficient mice were transplanted into chemically-induced diabetic recipients, and graft survival was assessed by monitoring blood glucose levels. Islet grafts deficient for TLR4, or for TLR signalling molecules TRIF and MyD88, had similar graft failure rates to islet grafts from wild-type controls, indicating that islet allograft rejection occurs independently of donor TLR signalling in mice.

To determine whether lack of TLR signalling via TRIF and MyD88 affects normal beta-cell function, mice deficient for TRIF or MyD88 were assessed for glucose tolerance, insulin sensitivity and in vitro glucose-stimulated insulin secretion. As TLR-induced inflammation has been demonstrated to enhance insulin resistance and diabetes-associated inflammation, lack of TLR signalling under non-pathological conditions may enhance beta-cell function. Interestingly, mice lacking TRIF demonstrated impaired glucose tolerance, hyperglycemia, hyperinsulinemia and impaired glucose-stimulated insulin secretion. Mice lacking MyD88 had similar glucose tolerance and insulin sensitivity to littermate controls, but displayed a mild impairment in glucose-stimulated insulin secretion.

The results of this thesis demonstrate that while TLR signalling is not likely to be essential for islet allograft rejection, TLR signalling may contribute to normal beta-cell function.
function. These findings point to a previously unrecognized role for TLR signalling in glucose homeostasis.
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARHGEF19</td>
<td>Rho guanine nucleotide exchange factor 19 (also known as WGEF)</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFRI</td>
<td>Child and Family Research Institute</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphodiester-guanine oligodeoxynucleotides</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Chemokine C-X-C motif ligand 10 (also known as IP-10)</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Danger associated molecular patterns</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosurea</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatic nuclear factor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of κB kinase</td>
</tr>
<tr>
<td>I</td>
<td>Insulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R1</td>
<td>Interleukin-1 receptor, type 1</td>
</tr>
<tr>
<td>IL-1R2</td>
<td>Interleukin-1 receptor, type 2</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-γ inducible protein 10</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intra-peritoneal glucose tolerance test</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia/reperfusion injury</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide (also known as endotoxin)</td>
</tr>
<tr>
<td>LY96</td>
<td>Lymphocyte antigen 96</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor like protein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Encoded by Ly96 gene</td>
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<tr>
<td>MECA-32</td>
<td>Mouse endothelial cell antibody 32</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MKK</td>
<td>Mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>MLDS</td>
<td>Multiple low dose streptozotocin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NAIP2</td>
<td>NLR family, apoptosis inhibitory protein 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>Gene encoding homeobox protein NKX2.2</td>
</tr>
<tr>
<td>Nkx6.1</td>
<td>Gene encoding homeobox protein NKX6.1</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide binding domain, leucine rich repeat containing proteins</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family, pyrin domain containing 3</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic mouse</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic duodenal homeobox 1</td>
</tr>
<tr>
<td>PI</td>
<td>Proinsulin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PolyI:C</td>
<td>Polynucleotide:polycytidylic acid</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptors for advanced glycation end products</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor interacting protein-1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SIGIRR</td>
<td>Single Ig IL-1 receptor-related molecule</td>
</tr>
<tr>
<td>SLC30A8</td>
<td>Gene encoding zinc transporter ZnT8</td>
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<tr>
<td>SLC39A10</td>
<td>Gene encoding zinc transporter Zip10</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TBK-1</td>
<td>TRAF-family member associated NF-κB activator binding kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor receptor associated factor 6</td>
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<tr>
<td>TRAM</td>
<td>TRIF related adaptor molecule</td>
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<td>TRIF</td>
<td>Toll/IL-1 receptor domain containing adaptor inducing interferon-β</td>
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<tr>
<td>WT</td>
<td>Wild-type mouse, C57BL/6</td>
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</tbody>
</table>
Acknowledgements

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Dedication

To my parents, Stewart and Julie Hamilton.

To my husband, Stewart Hutton.
CHAPTER 1: INTRODUCTION

*Diabetes mellitus* is a condition characterized by hyperglycemia and impaired glucose tolerance as a consequence of insufficient insulin production and/or action. The etiology of diabetes remains to be fully understood, however disease pathology shares similarities across the multiple forms of diabetes. While a genetic predisposition seemingly plays a role in diabetes, research is now focused on previously unexplored avenues of disease pathology, including environmental factors and systemic inflammation. Elevated markers of inflammation are present in diabetes, and as the distinction between types of diabetes becomes blurred, inflammatory and immunological pathways are providing clues towards a common origin.

Once regarded as a relatively minor ailment in the developed world, diabetes has escalated to unprecedented levels worldwide, in both the developed and developing world, becoming one of the leading causes of premature morbidity and mortality [1]. The number of people affected worldwide by diabetes (primarily type 2 diabetes) is expected to rise from 171 million in 2000 to 366 million in 2030, paralleling an increase in the elderly population (greater than 65 years of age) [2]. Aside from the negative health outcomes associated with diabetes, the burden on health care systems worldwide is increasing, and the Canadian health care system is projected to spend over $19 billion in 2020 on diabetes alone [3]. Rates of diabetes are increasing at an alarming rate due to increasing obesity and ageing in the population, as well as increased urbanization in both the developed and developing world [1-3]. Diabetes is a global epidemic and control of this disease requires both social and economical change.

There are multiple types of diabetes with the most prevalent being type 1 and type 2 diabetes. Other types of diabetes include gestational diabetes, latent autoimmune diabetes in adults (LADA), mature-onset diabetes of the young (MODY) and diabetes associated with genetic syndromes, such as Down syndrome [4].

1.1 Type 1 diabetes

Type 1 diabetes is characterized by hyperglycemia and impaired glucose tolerance, and is also accompanied by polyuria, polydypsia and fatigue. This form of diabetes occurs as
the result of an autoimmune attack on pancreatic beta cells, resulting in an inability to produce sufficient levels of insulin and ensuing hyperglycemia. Triggered by islet autoantigens, autoreactive immune cells invade the islet and selectively induce apoptosis in beta-cells, resulting in the onset of diabetes symptoms [5]. Autopsy studies of patients with recent-onset type 1 diabetes have revealed that there is a reduction of 80-90% in beta-cell mass in type 1 diabetes [6, 7]. Type 1 diabetes affects 5-10% of all people diagnosed with diabetes in Canada, equating to approximately 200,000 Canadians, and onset occurs primarily in children and adolescents [3, 8].

Rates of type 1 diabetes vary across the globe with low rates in China and Venezuela and a high incidence of type 1 diabetes in Finland and Sardinia with 35 cases per 100,000 [9, 10]. Genetic susceptibility to type 1 diabetes is related to both major histocompatibility complex (MHC) and non-MHC genes. Susceptibility loci within the human leukocyte antigen (HLA) class II region on chromosome 6 carry the highest risk of disease development [11]. HLA haplotypes associated with a high risk for developing type 1 diabetes are DR3 (DRB1*0301 DQA1*0501 DQB1*0201) and DR4 (DRB1*0401 DQA1*0301 DQB1*0302). HLA-alleles can also protect against type 1 diabetes, with DQB1*0602 allele conferring protection in the presence of islet cell antibodies [11]. Susceptibility loci have also been mapped to regions within the promoter of the insulin gene as well as to genes for CTLA-4, PTPN22 and IL-12p40 [11].

There is a fragile balance between immune regulation and reactivity within the body, and innate immunity plays a role in controlling what type of adaptive response is generated in response to exogenous or endogenous antigen. Innate immunity refers to the cells and mechanisms responsible for defending the host from infection in a non-specific manner, while adaptive immunity refers to the specific responses aimed to eliminate a pathogen based on antigen presentation. Innate immunity is essential for the recruitment of immune cells to the site of infection, activation the complement cascade, identification and removal of a pathogen and activation of adaptive immunity through antigen presentation to T cells. Three effector T-cell subsets of CD4+ T cells have been discovered: T helper (Th) 1, Th2 and Th17 [12]. Th1 responses are elicited in response to intracellular bacteria and viruses, and cytokines IFN-γ, TNF-α and IL-12 propagate the Th1 response. Parasitic worms (helminthes) drive a Th2 response, which is propagated through the secretion of IL-4, IL-5,
IL-13 and IL-25. The Th17 subset responds to extracellular bacteria and fungal infections, resulting in the secretion of IL-6, IL-17 and granulocyte colony-stimulating factor (G-SCF) [12, 13]. The balance of these CD4$^+$ T-cell subsets is dependent upon the nature of the antigen presented, the activation state of the antigen-presenting cell (APC) and cytokines secreted. Uncontrolled Th1 and Th17 responses can lead to autoimmunity whereas inappropriate Th2 activity can lead to allergy and asthma. Regulatory T-cells act to control Th1, Th2 and Th17 immune responses and prevent aberrant inflammation. Regulatory T cells are naturally present in the CD4$^+$ T-cell population; however, they can also be induced in the presence of TGF-β [12]. Forkhead box P3 (FoxP3) is a transcription factor expressed by a subset of regulatory T cells, and is responsible for regulating the suppressive nature of regulatory T cells [12]. These cells represent a minor but essential subpopulation of CD4$^+$ T cells.

Autoimmune destruction occurs when tolerance to self-antigen is broken, and inflammation outweighs regulation. While regulatory T cell numbers are similar in control and type 1 diabetic patients, suppressive action of regulatory T-cells from type 1 diabetic patients is decreased, leading to uncontrolled autoimmunity [14-16]. Autoimmune destruction is a coordinated event, initiated by the presentation of beta-cell autoantigens by APCs, such as glutamic acid decarboxylase and insulin, to CD4$^+$ T cells within the draining lymph nodes [5]. These immune cells then secrete an array of cytokines, such as IL-2, IL-1β, IFN-γ and TNF-α, propagating further inflammation and the migration of peripheral beta-cell specific CD8$^+$ T cells to the islet [5, 17]. Beta-cell apoptosis is initiated by beta-cell specific, cytotoxic CD8$^+$ T cells and over time, beta-cell mass is depleted [5]. Cytokines released by CD4$^+$ T cells can contribute to beta-cell injury through the activation of apoptotic cascades, such as the TNF-α/TNF-receptor 1 cascade, while T effector cells induce apoptosis through Fas-Fas ligand interactions and the perforin/granzyme system [18].

Beta-cell mass is dynamic and regulated by beta-cell replication, neogenesis and apoptosis [19]. In type 1 diabetes, beta-cell replication and neogenesis cannot match the rate of autoimmune destruction, resulting in decreased beta-cell mass and insufficient supply of insulin. Meier et al. reported that beta cells can be found in 88% of patients with long-standing diabetes, and beta-cell apoptosis, but not beta-cell replication, was increased in these patients, indicating the presence of ongoing beta-cell replenishment potentially due to
neogenesis [20]. In two other studies by this group, beta-cell replication was found to be increased in one patient with recent onset type 1 diabetes while in a group of patients with recent onset type 1, there was no difference [6, 21]. Varying rates of beta-cell regeneration and apoptosis may explain the differences in beta-cell mass and progression of disease seen in patients with type 1 diabetes.

While concordance rates are higher in monozygotic versus dizygotic twins, approximately 50% of monozygotic twins are discordant for type 1 diabetes [22]. This points to a combination of environmental triggers and genetic susceptibility to generate beta-cell autoimmunity. Environmental toxins known to be diabetogenic include N-nitroso compounds, vacor, cycasin, alloxan, streptozotocin and bafilomycin [23]. These toxins either induce DNA damage or interfere with beta-cell function, causing beta-cell death and autoimmunity in genetically susceptible individuals. Suspected dietary triggers of beta-cell autoimmunity include wheat and cow’s milk [24]. Wheat exposure during infancy has been reported to increase the risk of islet autoantibody development, while cow’s milk exposure has been linked to increased risk of generating type 1 diabetes in humans [24, 25].

There is a strong body of evidence linking viral infection to the initiation or exacerbation of type 1 diabetes and several hypothetical linkage mechanisms have been proposed including bystander activation, molecular mimicry and interference of regulatory T cell action. Antibodies to coxsackie virus were reported to be significantly elevated in patients with type 1 diabetes [26]. Coxsackie virus was isolated from a patient with diabetic ketoacidosis, and inoculation of mice with this isolate (CB4) induced insulitis and hyperglycemia [27]. The NOD mouse is an autoimmune model of diabetes used to study type 1 with similarities to autoimmunity in humans, including the presence of autoantibodies and T-cell mediated insulitis [28]. Infection of coxsackie virus may exacerbate autoimmunity in the NOD mouse through bystander activation. Virus-infected beta cells induce an anti-viral immune response, resulting in the initiation and enhancement of immunity against beta cells, and the potential release of sequestered autoantigens [29, 30]. While the strongest evidence linking viral infection with autoimmune diabetes is with coxsackie virus, other viruses such as rubella and parvovirus may also play a role. Rubella virus may initiate beta-cell autoimmunity via molecular mimicry. Viral antigen epitopes may be structurally similar to beta-cell autoantigen glutamic acid dehydrogenase, activating cross-
reactive T cells to induce autoimmunity [29, 31]. Viruses can affect regulatory T cell function, and may disturb tolerance, resulting in increased autoreactive T cell activity [32]. Parvovirus Kilham rat virus up-regulates Th1 CD4+ and cytotoxic CD8+ T cells and decreases regulatory and Th2 CD4+ T cells, inducing diabetes in the young diabetes-prone BioBreeding (BB) rat [33].

Aside from genetic models of autoimmune diabetes such as the NOD mouse and BB rat, diabetes can also be induced using a multiple low-dose streptozotocin (MLDS) regimen. Streptozotocin (STZ) is a fungus-derived beta-cell specific toxin that enters the beta cell through the GLUT2 glucose transporter. High-dose STZ initiates massive beta-cell inflammation and necrosis, whereas MLDS induces predominantly apoptosis in a mouse beta-cell line in vitro [34]. In vivo MLDS induces insulitis, followed by hyperglycemia [35].

Lack of caspase-3, an essential protease in apoptotic pathways, protects mice from MLDS-induced insulitis and cross-priming of beta-cell specific T-cells required for the initiation of autoimmune diabetes [36]. T-cell function may be central to the effects of MLDS as nude, athymic mice do not develop MLDS-induced diabetes [37]. The efficacy of this method to generate diabetes does rely on genetic susceptibility as some mouse strains are resistant to MLDS-induced diabetes [38].

Current treatment of type 1 diabetes consists of manually monitoring blood glucose levels and administering insulin subcutaneously with a hypodermic needle or an insulin pump. With the discovery of insulin in 1922 by Banting and Best, treatment of diabetes drastically changed from an invariably fatal disease to a manageable condition [39]. Insulin therapy remains the most cost effective and efficient therapy for the majority of persons with type 1 diabetes. Insulin therapy is expensive, however, and hyperglycemic and hypoglycemic episodes are common amongst users. Hypoglycemia can induce confusion, fatigue, dizziness, seizures and comas, and rapid hypoglycemia can be fatal. Patients with type 1 diabetes who are particularly susceptible to hypoglycemic episodes are hindered from daily activities like driving a car. Ketoacidosis arises during hyperglycemia, causing a lowering of blood pH. While temporary hyperglycemia is seemingly asymptomatic, chronic hyperglycemia increases risk of diabetes-related complications. Meticulous glycemic control can substantially reduce the risk of complications such as neuropathy, nephropathy and cardiovascular disease [40, 41]; however, this is laborious and sometimes difficult to achieve.
with current technology. Islet transplantation, gene therapy, and artificial pancreases are examples of potential therapies designed to supply insulin in response to glucose, and potentially alleviate the use of both glucose monitors and insulin injections for people with type 1 diabetes.

1.2 Islet transplantation

Islet transplantation, currently the only alternative treatment available to persons with type 1 diabetes besides exogenous insulin therapy, involves the isolation of islets from cadaveric donors, followed by their transplantation via the portal vein into the liver [42]. In 1892, Minkowski performed the first recorded transplantation of pancreatic tissues, performing a subcutaneous transplant of autologous pancreatic tissue from a pancreatetomized dog [43, 44]. Two years later, scientists attempted to treat a 13-year old child with a subcutaneous transplant of pancreatic tissue from sheep; however, this treatment eventually failed due to intense immunological rejection [44, 45]. In the late 1960s and early 1970s, techniques for islet isolation and transplantation were refined including the advent of the collagenase-based islet isolation method, and by 1975 transplanted islets were able to reverse diabetes in non-human primates [42, 46, 47]. During the 1980s and early 1990s, autologous transplants of islets isolated from patients with pancreatitis proved that islet transplants could provide long-term metabolic stability, thus paving the way for development of this therapy for persons with type 1 diabetes [48, 49]. By 1992, 167 islet transplants had been performed worldwide, but despite improvements in islet isolation methods and immunosuppressive regimens, insulin independence remained low, ranging from 15 to 52% amongst transplant recipients from 1990-1992 [50].

In 2000, Shapiro et al. published a report that demonstrated stable metabolic control could be achieved in islet transplant recipients with a glucocorticoid-free immunosuppressive regimen, consisting of sirolimus, low-dose tacrolimus and daclizumab, named the Edmonton protocol [51]. Glucocorticoids have been shown to be detrimental to islet function and glucose homeostasis, and prior to the Edmonton protocol, immunosuppressive therapies for islet transplantation included their use [52-54]. Other new strategies introduced with the Edmonton protocol included the elimination of animal serum from human islet medium and the immediate transplant of islets following isolation [51]. One year after islet
transplantation, 82% of patients remained insulin independent; however, after 5 years this number decreased to approximately 10% [55, 56]. Between 9,000 and 11,000 islet equivalents per kilogram of body weight are required for insulin independence [51, 57]. However in transplant recipients, the number of functional islets decreases over time. Islet grafts are subject to numerous immune and non-immune challenges, such as alloimmunity, autoimmunity, ischemic reperfusion injury, oxidative stress and islet amyloid deposits, all of which impact the mass and function of the transplanted islet graft [58-62].

While islet transplantation remains a viable treatment for persons with type 1 diabetes, two challenges exist with this protocol, limiting the global use of this therapy: the requirement for lifelong immunosuppression and limited tissue supply for transplantation. Commonly used immunosuppressants are toxic for islets [63, 64] and therefore other methods for immune protection such as islet encapsulation and the cotransplantation of islets with immunoprotective cells, such as Sertoli cells, are under development [65-68]. Currently, multiple donors are normally required to generate sufficient tissue to normalize blood glucose in one diabetic recipient. Alternate sources of tissue will be required to provide sufficient islet tissue for the many persons with type 1 diabetes. Xenotransplants from pigs have proved to be highly effective in reversing diabetes in non-human primates, and neonatal porcine islets may be potentially suitable for humans in the near future with the development of pathogen-free pigs [69-71]. Neonatal porcine islets are an attractive source for islets as they are resistant to high glucose and cytokine-mediated islet dysfunction [72, 73]. The use of embryonic stem cells to generate islet-like clusters continues to advance, and multiple groups have achieved normoglycemia when testing stem cell derived insulin-producing cell clusters in a transplant model [74, 75]. In vitro methods to reprogram other cell types into beta cells is another potential source of insulin-producing tissue and transdifferentiation methods continue to develop [76, 77].

1.3 Graft rejection

Islet transplants must combat both autoimmunity and alloimmunity in order to survive and properly secrete sufficient insulin. Two signals are required to generate an immune response: first, antigen is presented through the major histocompatibility complex (MHC) on an antigen-presenting cell (APC) to the antigen specific T-cell receptor (TCR) on
a T-cell, followed by non-antigen specific costimulation [78-81]. Costimulation involves the engagement of CD80/CD86 molecules on the APC with the CD28 receptor on T-cells, as well as the interaction of CD40 and CD154 (CD40L) (Figure 1) [78, 80, 81]. These pathways initiate further costimulation necessary for T-cell differentiation and proliferation. Antigen presentation in transplantation can occur two ways: direct or indirect. Direct presentation occurs when donor APCs present directly to host T-cells, whereas indirect presentation requires host APCs to capture donor antigen, followed by presentation to host T-cells [78]. The MHC used to present exogenous antigen is MHC class II, which presents to CD4⁺ T cells. Depleting APCs in culture prior to transplantation can prolong islet allograft survival, pointing to the importance, and perhaps predominance, of direct antigen presentation in islet transplantation [78, 82]. Allogeneic islet grafts have been shown to primarily induce a CD4⁺ T-cell response, however CD8⁺ T cells may play a role in chronic islet allograft rejection, as long-term islet allografts were susceptible to damage in the presence of activated CD8⁺ T cells [83, 84]. CD8⁺ T cells may also play a role in impairing induced islet xenograft tolerance [85]. As Th1 CD4⁺ T cells and beta-cell specific cytotoxic CD8⁺ T cells act synergistically to induce beta-cell apoptosis in type 1 diabetes, recurrent autoimmunity is also thought to contribute to islet allograft destruction [78, 86]. Graft tolerance is the ultimate goal in islet transplantation, and islet allograft tolerance can be achieved by generating allogeneic chimeras with donor bone marrow tissue and MHC matched grafts [87-89]. Currently, this intervention has not been attempted in humans due to the risk of graft versus host disease and the requirement of robust immunosuppression [89].

Immunosuppressant therapy targets both T-cell costimulation and replication pathways. Calcineurin is essential for IL-2 production after binding of MHC to the TCR, and the calcineurin inhibitor tacrolimus (FK506) is commonly used as an immunosuppressant in islet transplantation [79]. Sirolimus (rapamycin) prevents T-cell replication by inhibiting mTOR, a protein kinase essential for cell replication, while mycophenolate mofetil (Cellcept) inhibits purine biosynthesis [64, 79]. While these drugs are effective at preventing T-cell expansion, most are toxic to beta cells, capable of increasing beta-cell apoptosis and reducing glucose stimulated insulin secretion [90, 91].
Figure 1: Antigen presentation and costimulatory pathways.
Antigen presentation takes place in the draining lymph nodes where naïve T cells are polarized. In direct antigen presentation, the antigen presenting cell (APC) comes directly from the donor, presenting to host T cells, while indirect presentation requires host APCs to travel to the graft, obtain peptide and then present to T cells in the draining lymph nodes. Adapted from [79].
Although multiple barriers exist for the use of islet transplantation as a standard treatment, it remains a promising therapy for type 1 diabetes and can likely improve the quality of life for patients with this disease.

1.4 Type 2 diabetes

Type 2 diabetes is characterized by hyperglycemia and impaired glucose tolerance as a result of beta-cell dysfunction and reduced beta-cell mass. Type 2 diabetes is associated with obesity and insulin resistance [3]. Fasting plasma glucose (7.0 mM or greater), casual plasma glucose (11.1 mM or greater) or 2-hour post oral glucose tolerance test plasma glucose (11.1 mM or greater) measurements are used to diagnose diabetes [3]. Impaired glucose tolerance usually precedes diabetes, and declining beta cell function and increasing blood glucose levels characterize the progression towards disease. Identifying and treating prediabetes, characterized by impaired fasting glucose levels and glucose intolerance, can help prevent type 2 diabetes. Type 2 diabetes accounts for 90% of all diabetes cases worldwide, and in Canada, it affects approximately 2 million people [1, 3, 8].

Similar to type 1 diabetes, type 2 diabetes has both a genetic and environmental component in disease etiology. While excess body weight represents a large risk factor for type 2 diabetes, many obese patients will not develop type 2 diabetes and some patients (10%) with type 2 diabetes have normal body mass [92]. These findings highlight that while increased caloric intake and a sedentary lifestyle contributes significantly to the generation of type 2 diabetes, genetic susceptibility is also required for disease development. Some ethnic minorities and homogeneous populations (e.g. Aboriginal Canadians, Micronesians and Pacific Islanders) have a higher susceptibility to type 2 diabetes [92, 93]. First-degree family members of persons with type 2 diabetes have a 3.5-fold increased risk in developing disease, while monozygotic twins have a 70-75% disease concordance rate and a 96% concordance rate for the development of abnormal glucose metabolism [92, 94, 95]. As type 2 diabetes is a multi-factorial disease, genome wide analyses have been employed to look for genetic variances in patients with type 2 diabetes. Type 2 diabetes-associated gene loci include \textit{KCNJ11, PPARG, HHEX, SLC30A8} and \textit{TCF7L2}, with the highest risk of developing disease associated with the transcription factor \textit{TCF7L2} [92, 96]. While a large proportion of confirmed type 2 diabetes single nucleotide polymorphisms impact beta-cell function, gene
variants affecting adiposity, insulin sensitivity, insulin and glucagon secretion could all be involved in the genesis of type 2 diabetes, making the search for candidate risk-associated genes complex [92]. Monogenic forms of diabetes (e.g. MODY) have been traced to variants in \textit{HNF4A} (MODY1), \textit{HNF1A} (MODY3) and \textit{HNF1B} (MODY5), but these variants have not been consistently associated with development of type 2 diabetes [92, 97].

The thrifty genotype and phenotype hypotheses may provide clues towards potential mechanisms for the increased susceptibility of certain populations to obesity and type 2 diabetes. The thrifty genotype describes a susceptibility to obesity and diabetes due to a genetic predisposition geared towards increased fat and calorie storage [98]. While historically necessary for coping during times of famine, this inherent storage capacity is now a liability in the presence of an unlimited food supply in developed countries, leading to increased obesity and type 2 diabetes. This may potentially explain the prevalence of type 2 diabetes in the Aboriginal Canadian population, which is 3-5 times higher than the rest of the Canadian population [93]. Fluctuations in disease prevalence across susceptible populations, and failure to discover thrifty alleles challenge the thrifty genotype hypothesis [98]. Instead, epigenetic factors in early life are being sought out under the thrifty phenotype hypothesis. The thrifty phenotype hypothesis suggests that metabolic capacity is programmed during gestation and early post-natal life in response to the environment, and this phenotype is maintained throughout adulthood [98]. According to this hypothesis, type 2 diabetes occurs in individuals whose metabolic capacity is low due to malnutrition during gestation and infancy, leading to a lack of proper metabolism in times of plenty. This hypothesis is supported by data associating low birth weight with the development of type 2 diabetes and metabolic syndrome [99]. A retrospective study revealed that Dutch adults born during famine times of World War II with a low birth weight demonstrate impaired glucose tolerance and elevated fasting insulin and proinsulin levels [100]. This hypothesis also provides explanations for fluctuations in obesity and type 2 diabetes corresponding with societal and nutritional wealth as exhibited by the Pacific Island of Nauru [98, 99, 101]. These studies suggest that the etiology of type 2 diabetes involves a similar path as type 1 diabetes, involving a combination of environmental triggers and genetic susceptibility.

The development of complications in type 2 diabetes, including cardiovascular and microvascular disease, is strongly related to glycemic control [102]. Initially presenting with
fasting hyperglycemia and impaired glucose tolerance, type 2 diabetes progresses over time to increased beta-cell apoptosis and significantly decreased beta-cell mass [103, 104]. The progression of this disease can be controlled with diet and exercise; however, insulin sensitizing agents such as thiazolidinediones are typically required to control hyperglycemia [3]. Other pharmacological agents used to control type 2 diabetes include metformin, which acts to reduce hepatic glucose production, and sulfonylureas, which increase beta cell insulin secretion [3]. Exendin-4 is an incretin mimetic, which is also used to improve glucose control in patients with type 2 diabetes. In severe cases of type 2 diabetes, insulin must be used to control glycemia levels. Beta-cell mass in persons with type 2 diabetes is decreased due to an increase in beta-cell apoptosis and beta-cell mass begins to decline early in the course of disease, upon presentation of impaired glucose tolerance [104]. Insulin resistance increases insulin demand, initiating beta-cell proliferation and expansion of beta-cell mass. Over time, however, the capacity of beta-cells to match the excessive insulin demand diminishes, and beta-cell function declines (Figure 2). Markers of beta-cell dysfunction include reduced glucose-stimulated insulin secretion, impaired proinsulin processing, changes in pulsatile and oscillatory insulin secretion and reduced secretion of islet amyloid polypeptide (IAPP) [103]. Islet dysfunction and loss of beta-cell mass have been attributed to several factors including inflammation [105], ER stress [106] and islet amyloid deposition [107].

1.5 Regulation of beta-cell mass

Beta-cell mass is regulated by the rate of formation of new beta cells from beta-cell neogenesis and replication, and by the rate of destruction from beta-cell apoptosis [19]. Embryonic beta-cell development is directed by the presence of essential transcription factors pancreatic duodenal homeobox-1 (PDX-1) and Pax4, and upon differentiation, mature beta-cells express high levels of PDX-1, Nkx6.1, Nkx2.2 and Pax6 [108]. Beta-cell neogenesis is thought to be the primary mechanism behind the expansion of beta-cell mass in the fetus, as the proportion of replicating beta-cells is very low in the human fetus [108]. Beta-cell replication has been proposed as the primary mechanism for maintaining beta-cell mass during adulthood [109, 110]. Prolonged malnutrition during the late fetal and early postnatal phase in rats impairs beta-cell development but not beta-cell proliferation [111]. Beta-cell proliferation cannot restore sufficient beta-cell mass as re-nourished rats have a 35%
Figure 2: The dynamics of beta-cell mass in persons with type 2 diabetes.

As an individual progresses from normal to impaired glucose tolerance to type 2 diabetes, beta cells attempt to compensate for increased insulin demand, but eventually fail, likely due at least in part to increased beta-cell apoptosis and decreased beta-cell mass. Adapted from [105].
decrease in beta-cell mass in adulthood, indicating that fetal beta-cell expansion is essential for establishing adequate beta-cell mass [111]. The decreased beta-cell mass observed in fetal malnutrition is due to a decrease in beta-cell number rather than beta-cell size. Between birth and adulthood, beta-cell mass is thought to expand primarily by increasing beta-cell size rather than number [109]. Therefore, an equilibrium exists such that beta-cell neogenesis during development sets the lower threshold for the number of beta cells for sufficient beta-cell mass, and beta-cell proliferation and size expansion maintains beta-cell mass throughout adolescence and adulthood.

The role of neogenesis in regulation of beta-cell mass in adulthood remains to be fully elucidated. Fetal islet precursors express the same cytokeratin protein (CK19) as exocrine ducts, and these precursors may be active in adult life. In a subtotal rodent pancreatectomy model, neogenesis of beta cells from duct-like precursors has been suggested to be the primary mechanism behind beta-cell regrowth, whereas other groups have determined beta-cell proliferation as the principal mechanism in this model [112-114]. The ability of beta cells to replicate may decrease over time, as beta-cell senescence has been reported to occur in diet-induced type 2 diabetes in mice [115]. Without a compensatory increase in beta-cell replication, and in the presence of continued beta-cell apoptosis, there is a net decrease in beta-cell mass, inducing hyperglycemia (Figure 3).

While beta-cell apoptosis in type 1 diabetes is primarily due to autoimmune destruction, beta-cell apoptosis in type 2 diabetes is thought to result from endoplasmic reticulum (ER) stress. The ER of the beta cell is constantly synthesizing and processing insulin, and when insulin demand exceeds the capacity of the beta cell, misfolding of proteins occurs, activating the unfolded protein response and initiating ER stress. ER stress can initiate various pathways, leading to a reduction in protein translation as well as apoptosis [116, 117]. The transcription factor CHOP is capable of initiating beta-cell apoptosis in response to ER stress, and targeted gene disruption of CHOP delays ER stress-induced diabetes [118]. The Akita mouse develops diabetes due to a spontaneous mutation in the insulin 2 gene, which leads to misfolded insulin, increased ER stress, beta-cell apoptosis and decreased beta-cell mass [117, 119].

Hyperglycemia and hyperlipidemia can also initiate oxidative stress, by increasing mitochondrial activity, leading to the production of reactive oxygen species (ROS) [120].
Figure 3: Changes in beta-cell mass, replication and apoptosis over time in a normal, non-diabetic obese or type 2 diabetes adult.

Rates of beta-cell replication and apoptosis change over time, resulting in a net change in beta-cell mass in response to obesity and diabetes. Beta-cell mass increases in response to increased insulin demand by increasing beta-cell replication. However, in those susceptible to type 2 diabetes, beta cells cannot continually adapt to increased insulin demand, and beta-cell apoptosis increases, resulting in a net decrease in beta-cell mass. Adapted from [121].
Beta cells express low levels of anti-oxidant enzymes and are therefore very susceptible to oxidative stress [117, 120]. Consequences of oxidative stress include suppression of insulin biosynthesis, reduction in PDX-1 DNA binding capacity and decreased insulin secretion [122]. IAPP is co-secreted with insulin, and IAPP aggregation is associated with type 2 diabetes and islet dysfunction [123]. Impaired processing of IAPP can initiate beta-cell death and amyloid formation; however, the toxic species responsible for IAPP-induced beta-cell death remains to be determined [123-125]. Interestingly, this hallmark pathology of type 2 diabetes, is also seen in human islet transplants [61, 126].

Each of these pathogenic regulators of beta-cell mass can also contribute to the deterioration of beta-cell function and this initiates a cascade of events leading to an eventual insufficient beta-cell mass and hyperglycemia. There is a proposed equilibrium between beta-cell mass and function and a decline in one area will eventually stress the other, resulting in subsequent deterioration [127]. Beta-cell mass and function are highly interrelated and perhaps in diabetes, insult to either or both of these initiates a decline, resulting in hyperglycemia. The cause for this initial insult remains to be determined; however, altered regulation of inflammation is one of many plausible candidates.

1.6 Inflammation and diabetes

While type 1 and type 2 diabetes have seemingly different initiating mechanisms, both diseases result in decreased beta-cell mass and hyperglycemia. The mechanisms underlying beta-cell failure and loss in islet transplantation and type 2 diabetes may be similar, as in both scenarios islets are exposed to high metabolic demand and toxic conditions due to hyperglycemia, inflammation and islet amyloid. Inflammation plays a key role in mediating the decrease in beta-cell mass and hyperglycemia seen in both type 1 and 2 diabetes and therefore, diabetes is now viewed as both a chronic metabolic and inflammatory syndrome. High levels of circulating inflammatory cytokines such as IL-6, IL-1β and TNF-α are associated with obesity and diabetes [105, 128, 129]. White adipose tissue and the liver are major sites of pro-inflammatory cytokine secretion [129], although islets are also capable of secreting cytokines [130]. In a glucolipotoxic environment, human and mouse islets have impaired glucose-stimulated insulin secretion, and produce pro-inflammatory mediators such as IL-6, IL-8 and G-CSF [130]. Interleukins and free fatty acids induce serine (instead of
tyrosine) phosphorylation of insulin receptor substrates (IRS) which inhibits insulin action in peripheral tissues [131].

The balance between pro- and anti-inflammatory cytokines is important in the development of type 2 diabetes. Circulating levels of IL-6 and IL-1β, as well as the IL-1 inhibitor IL-1 receptor antagonist (IL-1Ra) are thought to play a role in disease pathology [131]. Interestingly, white adipose tissue is an important source of IL-1Ra in obesity and diabetes, and increased levels of IL-1Ra in obesity may contribute to insulin resistance [132, 133]. Mice lacking IL-1Ra have impaired lipid accumulation and are resistant to diet-induced obesity, indicating that IL-1 may play a role lipase activity [134]. Treatment of Goto-Kakizaki (GK) rats, a rodent model of non-obese type 2 diabetes, with IL-1Ra improves insulin sensitivity and decreased hyperglycemia [135]. Mice lacking the IL-1 receptor type 1 (IL-1R1) acquire mature-onset diabetes, marked by decreased glucose tolerance and insulin sensitivity, indicating that IL-1 action is also associated with increased insulin sensitivity [136].

Leptin, a glucose-regulatory hormone primarily synthesized by adipose tissue in proportion to fat mass, acts on the hypothalamic region of the brain and in peripheral tissues, including beta cells, to increase energy expenditure, decrease appetite and suppress insulin secretion [137]. Hypothalamic IL-1β levels are increased upon injection of leptin and leptin is unable to suppress food intake in II-1r1−/− mice, demonstrating that IL-1β is important for mediating leptin action [138]. Similar to the II-1r1−/− mice, II-6−/− mice develop mature onset diabetes and do not respond to leptin treatment [139]. The II-1−/− II-6−/− double knockout mouse develops obesity at a young age while the II-6−/− II-1ra−/− double knockout mouse was observed to be lean, potentially due to excess IL-1 signalling which promotes leanness independent of IL-6 [140]. Beta-cells are capable of producing IL-1β in a hyperglycemic environment, and IL-1β can induce beta-cell apoptosis [141, 142]. Human and murine islets are susceptible to impaired proinsulin processing after exposure to proinflammatory cytokines, implicating a direct role for inflammatory cytokines in beta-cell dysfunction and elevated proinsulin to insulin ratios seen in type 2 diabetes [143, 144]. In contrast, low levels of IL-1β have been observed to be beneficial for beta-cells as IL-1β can induce beta-cell proliferation in human islets [145]. IL-6 also has seemingly beneficial effects on glucose homeostasis as IL-6 infusion increases insulin stimulated glucose disposal during a
hyperinsulinemic-euglycemic clamp in humans [146]. IL-6 is increased during exercise and increases fatty acid oxidation [146, 147]. This combined evidence suggests that baseline, physiological levels of inflammatory cytokines such as IL-1 and IL-6 are important for regulating glucose homeostasis and lipid metabolism. However, when pro-inflammatory and anti-inflammatory cytokine levels become unbalanced, insulin resistance and diabetes can ensue.

Inhibiting inflammation can largely reverse the metabolic phenotype of diabetes. Treatment with salicylates improves hyperglycemia, hyperlipidemia and insulin resistance by inhibiting IKK-β activation of NF-κB, preventing a proinflammatory response [148, 149]. Targeting inflammatory pathways can also directly improve beta-cell function. Treatment of type 2 diabetes patients with recombinant IL-1Ra not only improved glycemic control but also decreased circulating IL-6 levels and the proinsulin to insulin ratio, indicating improved beta-cell function [150].

Establishing which inflammatory pathways and what cell types are involved in inducing beta-cell dysfunction in diabetes is crucial to advancing therapy. Resident macrophages and dendritic cells in both adipose and islet tissue are known to regulate much of the inflammation seen in these tissues. Obesity and type 2 diabetes are strongly correlated with an increase in macrophage infiltration in adipose and islets [130, 151]. Inflammation triggered by pathogen invasion, hyperglycemia and hyperlipidemia activates common inflammatory pathways, including JNK and IKK kinases. Activation of JNK and IKK results in the production of inflammatory cytokines as well as direct inhibition of insulin signalling, propagating the inflammatory state [151].

Although the majority of obese people have elevated free-fatty acids and profound insulin resistance, many will never develop type 2 diabetes. Similarly, genetic susceptibility does not always confer type 1 diabetes, and environmental triggers are thought to explain the high discordance rates of beta-cell autoimmunity between genetically identical twins. Therefore, there is a switch or trigger event in those that develop diabetes leading to the generation of an inflammatory milieu, followed by beta-cell dysfunction and apoptosis. My studies were directed towards examining various inflammatory mediators and their role in both inflammation and diabetes.
1.7 Toll-like receptors

While the list of inflammatory mediators continues to grow, one family of receptors has been widely implicated in the development of diabetes-associated inflammation: Toll-like receptors (TLRs). TLRs have been implicated in numerous inflammatory diseases such as atherosclerosis, systemic lupus erythematosus and rheumatoid arthritis [152-155]. TLRs are a family of pattern recognition receptors expressed on multiple cell-types including hematopoietic cells [156], endothelial cells [157], adipocytes [158], and pancreatic islets [159-161]. Homologues of a receptor initially discovered in Drosophila, TLRs are highly conserved pattern recognition receptors responsible for detecting markers of microbial pathogens such as lipopolysaccharide (LPS), double-stranded RNA, and flagellin (Table 1). TLRs are part of the innate immune system, which consists of non-specific host defense mechanisms against infection, such as the complement cascade and cytokine secretion. The innate immune system is responsible for responding to pathogens in a generic way. TLRs are capable of activating the adaptive immune system, which then confers long-lasting immunity.

The role of TLR activation in polarizing adaptive immune responses has evolved into a central component of the Danger Model, a model for the initiation of immune responses. Prior to the Danger Model hypothesis by Dr. Polly Matzinger, immune responses were thought to be initiated due to the foreign nature of an antigen [162]. The Danger Model does not assume that discrimination between self and non-self is the critical element to the initiation of an immune response [162, 163]. Instead, this model dictates that damage rather than strictly foreign material is the triggering factor [162]. Pattern recognition receptors (e.g. TLRs) are responsible for sensing these “danger signals” or danger associated molecular patterns (DAMPs), which are associated with cellular damage and necrosis (e.g. fibrinogen, heat shock proteins). Pathogen associated molecular patterns (PAMPs) are antigens derived from exogenous pathogens such as bacteria, virus or fungi. Some TLRs are capable of sensing DAMPs (e.g. TLR2, TLR4), and their sensitivity to a vast array of ligands is essential for controlling inflammation and tolerance. The sources of endogenous danger signals include necrotic and apoptotic cellular debris, oxidized lipids, proteins and nucleic acids, stress-induced cellular factors and extracellular matrix proteins [164]. Saturated free fatty acids (FFAs) stimulate IL-6 and TNF-α production via TLR4 in a dose-dependent
manner in macrophages while the polyunsaturated fatty acid docosahexanoic acid (DHA) blocks saturated FFA-induced TNF-α expression [165]. Interestingly, some TLRs have a high affinity for endogenous ligands (e.g. TLR2, TLR4) while others do not (e.g. TLR5) (Table 1). Other signalling pathways exist to activate inflammatory pathways in response to endogenous danger signals. Aside from activating NF-κB in response to endogenous stimuli, receptors for advanced glycation end products (RAGE) may also act in concert with TLR2 and TLR4 to initiate a proinflammatory response [164, 166].

**Table 1: Exogenous and endogenous TLR ligands.**

Adapted from [156, 165, 167-171].

<table>
<thead>
<tr>
<th>TLR</th>
<th>Exogenous Ligands</th>
<th>Endogenous Ligands</th>
</tr>
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<tbody>
<tr>
<td>TLR2</td>
<td>Diacylated (TLR6) or triacylated (TLR1) lipoprotein, atypical LPS, peptidoglycan, glycolipids</td>
<td>Necrotic cells, HSP70, HMGB1, minimally modified LDL</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral double-stranded RNA</td>
<td>Double stranded RNA from necrotic cells</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, glycoinositol phospholipids</td>
<td>Saturated fatty-acids, HMGB1, hyaluronan, HSP60, fibrinogen, heparan sulfate</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td></td>
</tr>
<tr>
<td>TLR7/8</td>
<td>Imidazoquinolines, viral-derived single-stranded RNA</td>
<td>Single-stranded RNA from dead or dying cells</td>
</tr>
<tr>
<td>TLR9</td>
<td>Bacterial CpG DNA, viral DNA</td>
<td>Chromatin-IgG complexes, HMGB1</td>
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TLRs possess distinctive leucine-rich repeats in the extracellular domain and have a cytoplasmic domain highly similar to the IL-1 receptor (IL-1R). Ten TLRs have been identified in humans; 13 have been identified in mice [172]. TLRs are expressed on the plasma membrane and in endosomes, and they signal via two intracellular molecules: myeloid differentiation primary response gene 88 (MyD88) and Toll/IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF). MyD88 is used by all TLRs with the exception of TLR3, which uses TRIF. MyD88 is also an essential signalling adaptor for the IL-1 receptor. TLR4 is unique in that it is the only TLR to have a bifurcated signalling pathway, using both TRIF and MyD88 (Figure 4). TLR4 signals as a homodimer complex with MD-2 and CD14, whereas TLR2 heterodimerizes with either TLR1 or TLR6 to
recognize either tricacylated or diacylated lipoproteins, respectively [170]. CD36 acts as a facilitator for diacylglyceride detection with the TLR2/6 complex [173].

Upon TLR activation, MyD88 recruits downstream signalling molecules IL-1 receptor associated kinase (IRAK)-1 and IRAK-4, which in turn activate TRAF6, leading to the activation of p38 mitogen-activated protein kinases (MAPK), c-Jun amino-terminal kinases (JNKs) and NF-κB (Figure 4) [156]. Activation of NF-κB leads to the production of inflammatory cytokines and expression of co-stimulatory molecules. In response to TLR7, TLR8 or TLR9 ligands, MyD88 can activate interferon regulatory factor (IRF) via TRAF6, leading to the production of interferon [174]. Similar to MyD88, TRIF also activates NF-kB through TRAF6, however TRIF activates IRF3 and IRF7 through TRAF3 and TBKI, stimulating IFN production. TRIF signalling can also activate receptor interacting protein 1 (RIP1), which is capable of activating NF-κB and inducing apoptosis via the Fas-associated death domain (FADD) [156, 174].

TLR signalling is regulated in multiple ways. LPS stimulation results in reduced cell surface expression of TLR4, MD-2 and CD14 [169]. TLR4, TLR2 and TLR7 ligands regulate NF-kB activation by selectively reducing expression of IRAK-1 [169, 175]. After continuous exposure to bacterial products or proinflammatory cytokines, an alternatively spliced form of MyD88 (MyD88s) is expressed which does not associate with IRAK-4, preventing downstream signal propagation [176]. IRAK-M is expressed in monocytes and macrophages in response to TLR agonists and it blocks the formation of the IRAK-1/TRAF6 complex [170]. TLR adaptor molecule TIRAP is targeted for proteosomal degradation by SOCS-1, creating a refractory period during prolonged TLR2 and TLR4 signalling [177]. SIGIRR (single Ig IL-1 receptor-related molecule) interacts with the IL-1R1, IRAK and TRAF6, preventing TLR and IL-1R induced NF-kB activation [178]. Tollip suppresses TLR2/4 activity by inhibiting IRAK activity, and overexpression of Tollip in intestinal epithelium may be important for gut tolerance [179, 180]. Each of these mechanisms serves to dampen TLR-induced inflammation, and impairment of these mechanisms can lead to TLR-associated inflammatory disease.
Figure 4: TLR signalling pathways.

TLRs sense both exogenous and endogenous ligands resulting in the activation of NF-κB and IRF, and the production of proinflammatory cytokines. Adapted from [169, 174].
Antigen presenting cells are responsible for bridging the gap between innate and adaptive immunity, and TLRs play a crucial role in directing T-cell polarization. TLR ligands in combination with cytokines are capable of initiating different T cell maturation states (i.e. Th1, Th2, Th17 or T-regulatory response) (Figure 5). T cells and B cells express TLRs and TLR ligation can enhance cell proliferation and cytokine production [181]. Regulatory T cells also express TLRs (TLR1, 2, 4-8) and TLR action can also directly modulate suppressive activity of regulatory T cells [182]. TLR5 ligation increases FoxP3 expression and enhances T effector cell suppressive action by regulatory T cells [183]. TLR2 has been directly implicated in mediating regulatory T cell expansion as TLR2-deficient mice have fewer regulatory T cells, and administration of TLR2 ligands to wild-type mice increases regulatory T cell numbers [184]. Triggering TLR2 results in a temporary suppression of T-regulatory action, and withdrawal of TLR2 agonist restores function [184]. TLR2 activity also increases CD25/IL-2 receptor expression on regulatory T cells, which is essential for driving IL-2 dependent regulatory T cell function and development [182]. This points to a role for TLRs in temporarily suppressing regulatory T cells during infection, allowing an inflammatory reaction in the presence of TLR agonist, and upon decline or withdrawal of TLR agonist, action of regulatory T cells is restored [182]. This impact of TLR action of regulatory T cell function in the presence of agonist may be a triggering factor in breaking immunological tolerance in chronic inflammation, autoimmunity and graft rejection. Therefore, targeting TLR pathways for disease therapy may prevent the initial onset of pathological inflammation and escalation towards a toxic inflammatory milieu as well as enhance immunological tolerance through regulatory T cell action.

1.8 TLRs and type 1 diabetes

Studies examining the role of TLRs in both type 1 and type 2 diabetes suggest that TLRs may play a role in both diseases. Autoimmunity in type 1 diabetes begins with an initial insult or lesion, which leads to the presentation of beta-cell autoantigen and the generation of an autoimmune response. Given the suspected role of environmental factors in type 1 diabetes, innate immunity may likely play a role in this first step.

The balance of effector and regulatory T-cell action is crucial in the development of type 1 diabetes. Inducing regulatory T cell expansion and function in a mouse model of type
Figure 5: Antigen-presenting cells bridge the gap between innate and adaptive immunity.

TLR activation by pathogens or endogenous danger signals on antigen presenting cells contributes to T cell polarization, activating a Th1, Th2 or Th17 response or altering suppressive action of regulatory T cells (Treg). Adapted from [169].
1 diabetes, the non-obese diabetic (NOD) mouse, can prevent diabetes development. Regulatory T cells can directly impact islet innate immunity by inhibiting cytokine production and preventing leukocyte recruitment [185]. However, as shown in transplantation studies, TLRs are capable of inhibiting regulatory T cell expansion and function [186, 187]. The order of events in the initial inflammatory process appears crucial to the outcome of disease.

TLRs have been directly implicated in the development of type 1 diabetes and beta-cell apoptosis. TLR3 and TLR5 are significantly upregulated in bone-marrow derived macrophages of newly diabetic NOD mice [188]. Kim et al. demonstrated that in the absence of TLR2, NOD mice and wild-type mice that receive MLDS treatment are protected from developing autoimmune diabetes [189]. Also, TLR3 and its signalling axis have been implicated in beta-cell apoptosis in response to double-stranded RNA and interferon-gamma [190, 191]. TLR2 and TLR4 expression in monocytes is up-regulated in patients with type 1 diabetes [192]. Wen et al. found that the TLR3 ligand poly(I:C), a mimic of viral double-stranded RNA, is capable of triggering type 1 diabetes in B6/RIP-B7.1 mice, a model in which the T-cell co-stimulatory molecule B7.1 (CD80) is expressed on beta cells [159]. Conversely, bacterial products such as LPS, a TLR4 agonist, were not able to induce diabetes development in the same model, affirming a viral trigger for T-cell mediated beta-cell destruction in this model.

TLR/IL-R signalling via MyD88 was recently implicated in the generation of diabetes in the NOD mouse, as MyD88⁻/⁻/NOD mice are protected from developing diabetes [193]. The proposed mechanism behind this protection was thought to relate to gut flora as MyD88-deficient NOD mice develop robust diabetes in a germ-free environment. Interestingly, TLR4 or TLR2-deficient NOD mice develop diabetes at the same rates compared to controls, speaking against a role for these TLRs in this mechanism. Lack of MyD88 leads to changes in the composition of gut flora in NOD mice, and this change may confer protection against environmental or microbial triggers of diabetes. Prediabetic NOD mice have increased intestinal permeability and infection with an enteric bacterial pathogen (Citrobacter rodentium) can accelerate the development of insulitis [194]. Increased intestinal permeability has also been observed in patients at risk for type 1 diabetes and other autoimmune diseases, allowing for greater exposure of the intestinal immune system to
antigens [195, 196]. This increased intestinal permeability may permit inappropriate immune reactions to food and microbial-derived antigens that would typically go unnoticed in a tolerant gut, leading to a breadth of triggers for autoimmunity [196].

While the initial trigger for TLR activation in type 1 diabetes remains unknown, the aforementioned studies clearly indicate that TLRs and innate immunity may play a role in generating autoimmunity.

1.9 TLRs and islet transplantation

TLR activation is capable of directing T-cell polarization towards a Th1, Th2 or Th17 response. Antigen presentation is an essential step in allograft rejection, and TLR activation through danger signals and endogenous ligands directs the T-cell response towards inflammation (Th1). Interestingly, MyD88 is important for Th1-driven immunity but not Th2. As lack of MyD88 does not fully diminish the Th1 response, a MyD88-independent pathway for Th1 immunity may exist [197, 198]. Preventing TLR activation on host and/or donor tissue may decrease Th1 T-cell polarization and subsequent allograft rejection.

In the transplantation setting, injury to donor tissue, through for example organ extraction, ischemia and reperfusion, and mechanical injury, increases TLR activation, priming the tissue for further infiltration of immune cells via the release of cytokines [199]. Several studies have examined how host versus donor TLR inactivation affects graft rejection. Deletion of both MyD88 and TRIF in donor skin allografts delayed full MHC-mismatched allograft rejection whereas deletion of only MyD88 in either donor or recipient was enough to prevent minor antigen mismatched skin allograft rejection [200, 201]. Conversely, lack of MyD88 in recipient or donor tissue was not enough to prevent MHC-mismatched skin and cardiac allograft rejection. However the ability of MyD88−/− dendritic cells to initiate a Th1 response was measurably lower, while Th2 immunity remained intact [202].

Among all of the TLRs, TLR4 has been proposed to play a potential role in allograft rejection due to the wide spectrum of endogenous ligands released during tissue injury. TLR4 has been implicated as a potential mediator of allograft rejection in liver, lung and kidney transplantation [203-205]. Furthermore, in human lung transplant recipients, grafts from donors with two loss-of-function TLR4 polymorphisms have improved long-term
survival [203]. Conversely, mice studies have shown that absence of TLR4 in recipients of MHC-mismatched skin allografts does not impact rejection [206]. These studies provide evidence that lack of TLR signalling may diminish allograft rejection. However, it remains to be determined whether TLR inactivation in the donor or host is more important, and whether TLR signalling plays only a minor role in the allograft response.

Transplantation tolerance is an ultimate goal in transplant immunology and TLRs may play an important role in preventing tolerance. Lung, intestine and skin transplants are more prone to acute rejection and less likely to develop tolerance under accepted regimens [207]. One hypothesis underlying this phenomenon is that lung, intestine and skin allografts are constantly exposed to environmental pathogens, which activate TLRs and initiate an immune response. Administration of the TLR9 agonist CpG at the time of transplant prevented tolerance in a cardiac allograft model while injection of TLR2, TLR3 or TLR4 agonists prevented tolerance in a skin allograft model [186, 208]. Administration of *Listeria monocytogenes* (LM) at the time of transplantation prevented allotransplant tolerance induced by CD154-blockade and donor specific transfusion therapy; however, this mechanism was MyD88 independent [209]. Regulatory T cells play an essential role in controlling allograft responses by neutralizing cytotoxic T lymphocytes. Multiple groups have determined that prolonged allograft survival is dependent upon regulatory T cells, and TLR activation can prevent regulatory T cell expansion and function [186, 187]. Administration of TLR9 agonist CpG to cardiac allograft recipients reduced intragraft CCL17 and CCL22 levels, preventing recruitment of T-regulatory cells. Another mechanism through which TLRs abrogate transplant tolerance is via the production of type 1 interferons. Thornley *et al.* observed that LPS and polyI:C, TLR4 and TLR3 agonists respectively, impair graft tolerance by inducing type 1 interferon production and activation of the type 1 interferon receptor, preventing deletion of cytotoxic T lymphocytes [198].

Islet grafts must undergo multiple processes before transplantation, all of which may induce intragraft inflammation. The islet isolation process can induce islet inflammation and apoptosis through the activation of JNK, p38 and NF-κB [210]. This predisposes islet grafts to inflammation upon engraftment, and also affects graft function in the immediate post-transplant period. The environment that the islet graft enters is a hostile one, including elevated markers of inflammation as well as hyperglycemia. Together, these conditions can
elevate TLR expression, and activation in both the islet graft and host may be important in exacerbating alloimmunity.

TLR4 is activated by exogenous ligand LPS, which is also a common contaminant in collagenase, an essential enzyme for islet isolation [165, 167, 211]. Interestingly, LPS negatively impacts graft survival by increasing intra-islet cytokine production and islet cell apoptosis, and LPS elimination can improve graft survival during the early post-transplant period [211, 212]. Controlling inflammation in the early post-transplant period is likely crucial to the viability and long-term function of allografts. TLR4 has been implicated in mediating ischemia/reperfusion injury (IRI) in kidney transplants as TLR4 mRNA is increased, and lack of TLR4 confers protection against IRI induced inflammation [213]. High-mobility group box 1 (HMGB1) is a damage-associated protein and secreted by transplanted islets. Neutralizing circulating HMGB1 recipients of sub-optimal, syngeneic islet transplants ameliorates hyperglycemia in sub-optimal islet transplants [214]. Also, islet grafts lacking TLR2 or receptor for advanced glycation end products (RAGE), established HMGB1 receptors, were protected from early islet graft loss [214]. This study demonstrates that innate immunity plays a role in early graft function, and early graft loss may not be due to purely alloimmunity. Therefore, eliminating TLR activity in islet allografts may not only ameliorate the development of alloimmunity, it may also enhance early graft function.

1.10 TLRs and type 2 diabetes

Fasting hyperglycemia and impaired glucose tolerance, combined with central adiposity and insulin resistance are hallmark characteristics of type 2 diabetes and are strongly associated with elevated markers of inflammation and immune cell infiltration. Targeting inflammation has assisted in controlling glycemia as well as improving beta-cell function in patients with type 2 diabetes. Inflammation can affect beta-cell function and survival, as well as insulin action in peripheral tissues and TLRs have been implicated in each of these areas.

A study by Rudofsky et al. revealed that type 2 diabetic patients heterozygous for the Asp299Gly and Thr399Ile TLR4 polymorphisms were less susceptible to diabetic neuropathy [215]. Interestingly, neither TLR4 polymorphism was detected in a Korean population,
indicating that ethnic differences could exist in the occurrence of TLR4 polymorphisms and may relate to type 2 diabetes susceptibility [216].

A recent study reported that the chemokine CXCL10, also known as IFN-γ inducible protein-10 (IP-10), is secreted at significantly higher amounts in islets isolated from patients with type 2 diabetes [160]. Interestingly, treatment of human islets with CXCL10 not only initiates beta-cell apoptosis, but also impairs glucose-stimulated insulin secretion. CXCL10 was observed to induce beta-cell apoptosis in a TLR4-dependent manner through a switching of Akt signals from proliferation to apoptosis. Mouse islets lacking TLR4 were protected from the CXCL10-mediated apoptosis and dysfunction [160]. Cultured islets isolated from mice lacking MyD88 secrete significantly less IL-6 and chemokine KC in response to palmitate and glucose, demonstrating that metabolic stress acts via MyD88 to initiate an inflammatory response [135].

The production of inflammatory cytokines leads to insulin resistance in peripheral tissues and the activation of JNK leads to serine-phosphorylation of IRS-1, down-regulating insulin signalling [217]. To determine if TLR activation of NF-κB and JNK mediated insulin resistance in vivo, multiple groups examined a Tlr4 knockout mouse model on a high fat diet [165, 218, 219]. Collectively they have shown that mice lacking TLR4 are protected from diet-induced insulin resistance. Mice lacking Tlr4 showed a decreased level of phosphorylated IκBα, intercellular adhesion molecule (ICAM)-1 and IL-6 mRNA compared to WT mice [218]. Lack of TLR2 signalling protected mice on a high-fat diet from insulin resistance, impaired glucose stimulated insulin secretion and islet inflammation [220]. In contrast, lack of TLR5 induces insulin resistance, hyperlipidemia, hyperphagia and glucose intolerance [221]. This phenotype was proposed to be related to the gut microbiota as transfer of the Tlr5−/− mouse gut flora to wild-type, germ-free mice induced the Tlr5−/− mouse phenotype.

A recent study examined the role of MyD88 in glucose homeostasis and reported that while mice lacking MyD88 have normal glucose tolerance and fasting blood glucose levels, they have decreased beta-cell mass compared to wild-type controls [222]. These findings provide evidence that TLR signalling may be important in the generation and/or replication of beta-cells. Interestingly, this group also reported that when treated with a MLDS regimen to induce beta-cell death, mice lacking MyD88 developed glucose intolerance. This finding
points to a role for TLRs in preventing beta-cell inflammation, dysfunction and apoptosis. As MyD88 is also the major signalling molecule for the IL-1 receptor, it remains to be determined whether these findings reflect loss of TLR signalling, or if they are a result of loss of action of other receptors [223].

Free-fatty acids induce a proinflammatory response completely dependent on MyD88 and partially dependent on TLR2 and TLR4 [224]. Stearate (C18:0), a common circulating free fatty acid in humans, significantly increased IL-1β and chemokine KC mRNA in wild-type islets, whereas islets lacking MyD88 were protected from this effect. Similarly, islets lacking TLR2 or TLR4 were protected from stearate-induced chemokine KC but not from IL-1β autostimulation [224]. Interestingly, islet TLR2 activity was partially dampened in the presence of IL-1Ra, providing evidence that IL-1R1 activation serves to amplify TLR-induced inflammation.

These studies provide evidence that TLRs may play a role in both beta-cell homeostasis and type 2 diabetes pathophysiology. While current studies have demonstrated a role for TLRs in inflammation and survival of beta-cells (Figure 6), it remains to be determined how TLR signalling affects glucose tolerance and beta cell function under physiological conditions.
Figure 6: High levels of circulating glucose and free fatty acids stimulate cytokine production via TLRs and the IL-1R, leading to islet dysfunction.

Chronically elevated glucose and free fatty acid (FFA) levels stimulate the production of NF-κB and inflammatory cytokines IL-1β, IL-6 and TNF-α. Glucose and FFAs activate NF-κB through multiple mechanisms including the formation of reactive oxygen species and activation of protein kinase C. IL-1β activates the IL-1R, promoting further IL-1β production, down-regulation of IL-1Ra and NF-κB activation. IL-1R activity amplifies TLR induced inflammation. Activation of NF-κB results in the production of IL-6 and TNF-α, which along with IL-1β promote increased cytokine production, macrophage infiltration, apoptosis and decreased insulin secretion. TLR activation of TRIF and MyD88 results in the activation of both pro-IL-1β and NF-κB [169]. Adapted from [225].
1.11 Study hypothesis and objectives

Diabetes manifests as a result of a decreased functional beta-cell mass, resulting in hyperglycemia. The role of inflammation in initiating beta-cell dysfunction and apoptosis in diabetes seems undeniable; however, the mechanisms behind the generation of inflammation in diabetes remain to be determined. Understanding the role of innate inflammatory pathways is central in resolving this mechanism. Innate immune pathways are essential for the development and polarization of adaptive immune responses, and TLR activation is a key process in this process. Aberrant TLR activation in response to endogenous ligands or danger signals could be the triggering mechanism behind the disruption of self-tolerance and regulated inflammation in autoimmunity and diabetes.

The overall objective of this thesis was to determine the role of TLRs in islet allograft rejection and normal beta-cell function.

The overall hypothesis for this thesis is that TLR signalling is essential for islet allograft rejection and is detrimental to normal beta-cell function and glucose homeostasis.

This thesis addressed three separate objectives:

**Objective 1:** To determine the role of TLR signalling in full MHC-mismatched islet allograft rejection.

**Objective 2:** To determine the role of the TLR signalling molecule TRIF in normal beta-cell function and glucose homeostasis.

**Objective 3:** To determine the role of the TLR signalling molecule MyD88 in normal beta-cell function and glucose homeostasis.

As diabetes affects millions worldwide, it is essential to establish the mechanisms of disease initiation and progression. While the way of life of persons with diabetes continues to improve with advances in insulin delivery, no cure exists for this devastating disease, and understanding the role of TLR-mediated inflammation may shed new light on disease mechanisms. Establishing the role of TLRs in islet transplantation and diabetes may lead to new therapeutics to prevent islet allograft failure and the progression of diabetes.
CHAPTER 2: METHODS AND MATERIALS

2.1 Animals

BALB/cJ, C57Bl/6J-Ticam1Lps2/J (Trif\(^{-/-}\)), C57BL/10ScNJ (Tlr4\(^{-/-}\)), B6;129S1-Tlr3\(^{tm1Flv}\)/J, C57BL/10J, C57BL/6J animals, 8-10 weeks old, were purchased from The Jackson Laboratory (Bar Harbor ME). C57BL/6-MyD88\(^{-/-}\) (MyD88\(^{-/-}\)) animals were obtained from Dr. Jan P. Dutz (Department of Dermatology and Skin Science, Faculty of Medicine, University of British Columbia) and were originally purchased from Oriental BioScience (Kyoto, Japan). C57BL/6-MyD88\(^{-/-}\)/Trif\(^{-/-}\) animals were generated in-house from Trif\(^{-/-}\) and MyD88\(^{-/-}\) animals and genotyped for expression of both mutant genes. B6;129S1-Tlr3\(^{tm1Flv}\)/J mice were backcrossed with C57BL/6J mice for 10 generations to generate mutant TLR3 mice on a C57BL/6 background (Tlr3\(^{-/-}\)). All animals were housed at the Child and Family Research Institute Animal Care Facility and maintained in compliance with Canadian Council on Animal Care guidelines. Trif\(^{-/-}\), MyD88\(^{-/-}\), Tlr4\(^{-/-}\), Tlr3\(^{-/-}\), MyD88\(^{-/-}\)/Trif\(^{-/-}\), C57BL/6 were bred in-house and genotyped accordingly for expression of mutant genes. Breeder and colony cages of MyD88\(^{-/-}\) and MyD88\(^{-/-}\)/Trif\(^{-/-}\) animals, and littermate controls, included drinking water supplemented with antibiotic, sodium sulfamethazine (1 mg/L; Sulfan 25% Solution, Vetoquinol, Lavaltri QC), to prevent infection.

2.2 Genotyping

To obtain genomic DNA, an ear punch sample was taken from weaned mice (4-6 weeks of age) and digested with Proteinase K (20 mg/mL; Gibco, Invitrogen Canada, Burlington ON) in SET buffer (20 mM Tris, 5 mM EDTA, 1% SDS) at 37°C while shaking at 1300 rpm. DNA was precipitated using 4.9 M NaCl/245 mM KCl and ice cold 95% ethanol, followed by resuspension in sterile ddH\(_2\)O. Polymerase chain reaction (PCR) primer sequences and thermocycler programs for Tlr3\(^{-/-}\), Tlr4\(^{-/-}\), and Trif\(^{-/-}\) animals were obtained from The Jackson Laboratory. PCR primers for genotyping MyD88\(^{-/-}\) animals were obtained from the laboratory of Dr. Jan P. Dutz (Table 2.1). Gene targets were amplified by PCR using the following program 94°C for 3 minutes, 35 cycles at 94°C for 30 seconds, primer annealing temperature for 1 minute and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were separated by gel electrophoresis on a 1.5% agarose gel (ultraPURE,
Gibco) with ethidium bromide (0.5 µg/mL; Sigma Aldrich, St Louis MO). To differentiate between wild-type and mutant PCR products from Trif<sup>−/−</sup> mice, PCR products were sequenced as mutant mice contain a single base pair deletion. PCR products for Trif<sup>−/−</sup> mice were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Mississauga ON) and sequenced by the Centre for Molecular Medicine and Therapeutics DNA Sequencing Core Facility (CFRI, Vancouver BC) on an ABI 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City CA)

**Table 2: PCR primer sequences for genotyping.**

<table>
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<th>Gene</th>
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<th>Reverse Primer (5’-3’)</th>
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<td></td>
<td></td>
<td>TGGCATGCCTCCATCATAGTTAACCC</td>
<td>ATCGCCTTCTATCGCCTTCTTGAAGC</td>
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<td>TRIF/Ticam-1</td>
<td>WT (152 bp) MUT (151 bp)</td>
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<td></td>
<td>TGCAAGTTTTCTATATGCAAG</td>
<td>CCTCCATTTTCAATACCTAG</td>
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<td>TLR3</td>
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<td></td>
<td>CTTGGGTGGAGGCTATTC</td>
<td>AGGTGAGATGACAGGAGATC</td>
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### 2.3 Islet isolation

Pancreata from Avertin-euthanized (Sigma) islet donors were perfused retrogradely via the pancreatic duct with collagenase (1000 units/mL type XI, Sigma) dissolved in Hank’s Balanced Salt Solution (Cat. 14185, Gibco), followed by digestion at 37°C (14 minutes static plus 1 minute of shaking by hand) and purification by the dextran-gradient method [226, 227] or by the filtration method [228]. Islets for transplantation were isolated using the gradient method while islets for in vitro experiments and RNA extraction were isolated using the filtration method. A dextran gradient was formed by resuspending islets in 1.10 g/mL of dextran (Sigma) in Ham’s F10 Medium (Gibco) and then applying 3 layers of less dense dextran (1.085, 1.075 and 1.045 g/mL) on top. Islets were then centrifuged at 1800 rpm for 20 minutes at 4°C and islets were picked at the interface of layers 1.085/1.075 g/mL and
1.075/1.045 g/mL. Islets were washed and incubated overnight in Ham’s F10 medium (Gibco) at 37°C. Prior to transplantation, islets were washed, counted and hand-picked into groups of 500 islets. To purify islets by filtration, tissue was filtered through a 70 µm nylon cell strainer (BD Biosciences, Mississauga ON) immediately after collagenase digestion. Islets were then hand-picked from the filtrate and contaminating exocrine tissue was removed. Islets were then incubated overnight in RPMI 1640 (Cat. 11875077, Gibco) plus 10% fetal bovine serum and washed prior to use for in vitro studies.

2.4 Islet transplantation

Under isoflurane anesthesia, islet grafts (500 islets/graft) were transplanted into the sub-capsular space on the left kidney of graft recipients previously rendered diabetic by intraperitoneal injection of STZ (250 mg/kg body weight; Sigma). Induction of diabetes occurred 3-5 days prior to transplantation of islet grafts and was confirmed by two consecutive blood glucose readings greater than 20 mM. Graft function was assessed by recipient blood glucose levels which were monitored daily for the first week following transplantation, followed by twice-weekly measurements until graft failure (return to hyperglycemia; ≥ 20 mM) or sacrifice. Graft failure was characterized by two consecutive blood glucose measurements greater than 20 mM. All islet grafts were excised, fixed in Z-Fix (Anatech Ltd., Battle Creek MI) and processed for histology.

2.5 Histology

All tissue samples for histology were fixed in Z-Fix (Anatech) for 4-6 hours, washed in 70% ethanol and then processed into paraffin sections in the Histology Core at the CFRI (Vancouver BC). Cut sections (5 µm) for immunohistochemistry were deparaffinized in xylene and hydrated through 95% and 70% ethanol to distilled water. Slides were stained with hematoxylin and eosin (H&E; Protocol, Fisher Scientific Canada, Ottawa ON), or immunostained for a variety of cellular targets (Table 3). Antigen retrieval was required for the majority of the primary antibodies used; 0.1 M sodium citrate or DAKO Target Retrieval Solution (Dako Canada, Mississauga ON) was used dependent on the antibody. For all immunofluorescence, slides were coverslipped using Vectashield with DAPI Mounting Medium (Cat. H1200, Vector Laboratories, Burlington ON). Sections were visualized using
a Leica DM4000B microscope and images were obtained using a Qimaging Retiga 1300i FAST camera and OpenLab 4.0.2 software (Improvision, Waltham MA).

Table 3: Antibodies and concentrations for immunofluorescence.

<table>
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<th>Target</th>
<th>Manufacturer</th>
<th>Concentration Used</th>
<th>Antigen Retrieval Method</th>
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<tr>
<td>Insulin</td>
<td>Dako (A0564)</td>
<td>1:100 – 1:500</td>
<td>Not required</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Dako (A0565)</td>
<td>1:75</td>
<td>Not required</td>
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<td>0.1 M sodium citrate</td>
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<td>MECA-32</td>
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<td>BrdU</td>
<td>Accurate Chemical (OBT0030)</td>
<td>1:300</td>
<td>0.1 M sodium citrate</td>
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</tbody>
</table>

2.6 Beta-cell mass and replication quantification

To calculate beta-cell mass, extracted pancreata were weighed prior to fixation. Serial sections were cut from five representative regions throughout the pancreas, separated by 100 µm. Following deparaffinization, sections from each region were immunostained with insulin antibody (1:500, Dako) followed by incubation with a biotinylated anti-guinea pig secondary antibody raised in goat (1:200; Jackson Immuno Research, West Grove PA). Insulin-positive tissue was visualized using liquid DAB (BioGenex, San Ramon CA), followed by a counterstain with Zymed hematoxylin (Invitrogen Canada). Sections were then visualized using an Aperio ScanScope GL (Aperio Technologies Ltd., Vista CA) and the insulin-positive ratio (insulin positive pixels/total pixels) was calculated using the Positive Pixel Algorithm with ImageScope software (Aperio Technologies). The insulin positive ratio was then combined with pancreas mass to determine beta-cell mass.

To calculate beta-cell replication, beta-cell BrdU (Cat. A2139 0005, AppliChem Inc., Darmstadt, Germany) incorporation was quantified. Drinking water supplemented with BrdU (1.0 mg/mL) was given to animals for 6 days, after which animals were euthanized, and pancreata were removed and fixed for histology. After paraffin processing, serial sections were cut from five representative regions as above. BrdU was immunostained using
an anti-BrdU antibody raised in rat (1:300, Cat. OBT0030, Accurate Chemical, Westbury NY), followed by incubation with Alexa 488-conjugated anti-rat secondary antibody, raised in donkey (1:200, Molecular Probes). Sections were simultaneously immunostained with insulin using the previously mentioned polyclonal insulin antibody (Dako) and Alexa 594-conjugated anti-guinea-pig secondary antibody raised in donkey (1:200, Molecular Probes). Slides were coverslipped using Vectashield with DAPI Mounting Medium (Vector Laboratories). Sections were visualized using a Leica DM4000B microscope and images were obtained using a Qimaging Retiga 1300i FAST camera and OpenLab 4.0.2 software (Improvision). BrdU positive nuclei and insulin positive cells were counted manually; islet area was obtained using Image-Pro (v.6.2.1.491, Media Cybernetics, Bethesda MD) image processing software. Beta-cell size comparisons were made using the ratio of the number of insulin positive cells to islet area.

To examine beta-cell rosettes, plasma membrane and endothelial cells were immunostained using E-cadherin (1:50, Cat. 610181, BD Biosciences, Mississauga ON) and MECA-32 (1:50, Cat. SC19603, Santa Cruz Biotechnology Inc.) antibodies, respectively. Slides were coverslipped with Vectashield with DAPI Mounting medium (Vector Laboratories) to visualize nuclei, and images were taken on an Olympus BX61 microscope using In Vivo Imaging Software (Olympus America Inc., Center Valley PA). Nuclei distances were calculated using Image-Pro (Media Cybernetics) processing software.

2.7 In vivo metabolic testing

Age-matched mutant and wild-type mice were fasted for four hours prior to the start of all metabolic tests (intra-peritoneal (i.p.) glucose and insulin tolerance tests) and measurements for blood glucose and plasma insulin. Basal blood glucose measurements were made using a glucometer (OneTouch, Burnaby BC). Blood was collected from the saphenous vein in EDTA coated microvette tubes (Sarstedt Inc., Montreal QC) and plasma was extracted after centrifugation. Insulin was quantified from plasma using a rat insulin ELISA (Crystal Chem Inc, Downers Grove IL) or an ultra-sensitive mouse insulin ELISA (ALPCO Diagnostics, Salem NH) and proinsulin was quantified using a mouse proinsulin ELISA (ALPCO Diagnostics). Plasma glucagon was measured using a glucagon radioimmunoassay kit (Cat. GL-32K, Millipore, Billerica MA). To measure glucose
tolerance, mice were injected with 50% dextrose (Sigma) at a dose of 1.5 mg dextrose/g of body weight. Blood glucose levels were measured at 15, 30, 60 and 120 minutes post-injection and a blood sample was taken for mice whose blood glucose rose above the sensitivity of the glucometer (>34 mM). Blood glucose concentration was then confirmed by assay (BioAssay Systems Inc., Hayward CA). To determine insulin sensitivity, mice were injected with 0.33 U/mL insulin (Novolin® ge Toronto; Novo Nordisk Canada, Mississauga ON) at a dose of 0.75 U/kg of body weight. Blood glucose levels were measured at 15, 30 and 60 minutes post injection. Blood glucose levels and area-under-the-curve (AUC) values were used to compare groups in both tests.

To compare metabolic responses on a high-fat diet, mice were given a 45% kcal high-fat diet (Cat. D12451, Research Diets Inc., New Brunswick NJ). Mice were given this diet for 12 weeks, and metabolic testing (IPGTT, ITT and blood sample collection) was performed at 4-week intervals over this period. Control mice were maintained on normal chow diet (4.5% kcal fat, Cat. 5053, PicoLab Rodent Diet 20, PMI Nutrition International), provided by the CFRI Animal Care Facility.

2.8 In vitro insulin secretion studies

Islets were isolated from euthanized mouse donors and were plated in triplicate for each condition (low (1.67 mM) and high (16.67 mM) glucose) with 20 islets/well in a 96-well plate. Islets isolated from three animals per genotype were pooled for each experiment. Islets were pre-incubated in Krebs-Ringer bicarbonate (KRB) buffer containing 10 mM HEPES (pH: 7.4), 0.25% BSA and 1.67 mM glucose for two hours at 37°C, followed by stimulation with KRB-1.67 mM or KRB-16.67 mM for one hour at 37°C. Media and islets were collected from each well to measure insulin secretion and content, respectively. Islets were lysed in 150 µL 5% acetic acid plus 1% BSA (Sigma) by boiling samples for 10 minutes, followed by centrifugation for 10 minutes at 12,000 rpm at 4°C. Insulin levels in islet extracts and media were determined using a mouse insulin ELISA (ALPCO Diagnostics). Total protein was calculated using a BCA Protein Assay Kit (Cat. 23225, Thermo Scientific, Fisher Scientific Canada).

The dynamics of insulin secretion were measured from isolated mouse pancreatic islets using a standard islet perifusion protocol [229], in the laboratory of Dr. J. Johnson.
(University of British Columbia). Islets were isolated as above, and incubated overnight in RPMI 1640 plus 10% FBS (Gibco). Groups of 100 size-matched islets were suspended with Cytodex microcarrier beads (Sigma) in 300 µL plastic chambers of an Acusyst-S perifusion apparatus (Endotronics, Minneapolis MN). Islets were perifused at 37°C and 5% CO₂ at 0.5 mL/min with a KRB buffer containing: 129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, 3 mM glucose and 5 g/L radioimmunoassay-grade BSA (Sigma). Prior to sample collection, islets were equilibrated in basal (3 mM glucose) conditions for 1 hour. Samples were frozen prior to analysis of insulin levels by specific radioimmunoassay (Millipore/Linco, Billerica MA).

2.9 Reverse transcriptase-PCR

RNA was extracted from aliquots of 150-200 islets isolated from age-matched animals following overnight culture using an RNeasy Mini Kit (Qiagen Inc.). Islet RNA was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). RNA samples (0.5 µg/mL) were converted to cDNA using SuperScript III Reverse Transcriptase Kit (Invitrogen Canada) and then analyzed for gene expression using cDNA primers (Table 4). Real-time RT-PCR was performed using Power SYBR Green PCR Master Mix Kit (Applied Biosystems Inc., Foster City CA) with a 7500 Fast Real Time PCR System (Applied Biosystems Inc.). Results were analyzed by comparing cycle threshold values and normalized to GAPDH expression as an internal control.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>GCTTTCACCTCTGCCTTCAC</td>
<td>AGGCGATACAAATTCCACCTG</td>
</tr>
<tr>
<td>MyD88</td>
<td>AAGGACAAACGGCGGAACCTTTT</td>
<td>GCCGATAGTCTGTCTGTTCATAGT</td>
</tr>
<tr>
<td>NAIP2</td>
<td>GGGAGCAACCACGGGAGGCT</td>
<td>GCTGGGCAGTCGGGACACCA</td>
</tr>
<tr>
<td>LY96</td>
<td>CGCTGCTTTTCTCCCATATTTGA</td>
<td>CCTCAGTCTTATGCAGGCTTC</td>
</tr>
<tr>
<td>ARHGEF19</td>
<td>AGGCCCAGATACCCATATCG</td>
<td>GCCTCTTCCATATGATACACCTG</td>
</tr>
<tr>
<td>GLUT2</td>
<td>TTGACTGGAGCCCTCTTGATG</td>
<td>CACTCTGCTCCAGCAATGATGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCGAGTCAAACGGATTGGTCGTAT</td>
<td>AGCCCTTCTCCATGGTGTAAGAC</td>
</tr>
</tbody>
</table>
2.10 Gene expression analysis

Genome wide analysis of islets from $Trif^{-/-}$ (n=4) and C57BL/6 (WT; n=4) mice was achieved through direct hybridization assay using an 8-sample, mouse Illumina Sentrix BeadChip array (MouseRef-8 v2.0 Expression BeadChip, Illumina Inc, San Diego CA) and was performed by the Genotyping & Gene Expression Core Facility (Centre for Molecular Medicine and Therapeutics, CFRI, Vancouver BC). Greater than 23,000 NCBI RefSeq genes were probed for using a 50-base gene-specific probe, which was hybridized to labeled nucleic acid derived from total RNA. RNA was isolated from islets as above from age-matched, 10-week old male mice. Gene expression of $Trif^{-/-}$ and WT islets was compared using a Differential Expression analysis algorithm on BeadStudio (version 3.1.3.0, Illumina). Hybridization controls were included in the array and results were compared to expected values to ensure the quality of the assay. Negative controls were included to control for background signal. All samples were normalized to the average background signal before comparison. Only those samples detected significantly above background were analyzed.

2.11 Flow cytometry

Spleens were excised and splenocytes isolated from age-matched animals in RPMI plus 10% FBS (Gibco). Splenocytes (1 x $10^6$) were cultured with or without LPS (0.1 mg/mL; Sigma) in 1 mL of RPMI containing 10% FBS. After 24 hours, the cells were washed and stained with fluorescent-conjugated monoclonal antibodies to CD40, CD86 and B220 conjugated to APC (CD40), PE (CD86) and PERCP (B220) (BD Biosciences Pharmingen Canada, Mississauga ON) to assess TLR activation (CD40 and CD86) and an antigen presenting cell population (B220). Flow cytometry analysis was performed on a FACS Calibur flow cytometer (BD Biosciences) using Cell Quest (BD Biosciences) and FlowJo (Tree Star Inc., Ashland OR) software.

2.12 Data and statistical analysis

Data presented are mean ± SEM (standard error of the mean). Statistical significance was determined using the Student’s t-test; $P<0.05$ was considered statistically significant. Data and statistical analysis were performed using Prism, version 4.0 (GraphPad Software Inc., La Jolla, CA).
CHAPTER 3: THE ROLE OF TLR SIGNALLING IN ISLET ALLOGRAFT REJECTION

3.1 BACKGROUND

The success and survival of islet allografts is dependent on both immunological and non-immunological factors. Immunosuppression is necessary to prevent graft rejection; however, the use of immunosuppressants jeopardizes graft function and does not prevent chronic graft failure [51, 55, 89]. Early graft failure is common, as islets are transplanted to an ectopic site and may not vascularize properly, resulting in a decreased islet graft mass [60]. Intra-islet inflammation and the formation of islet amyloid can also contribute to early graft failure, resulting in the need for top-up transplants to maintain sufficient glucose control in islet allograft recipients [60, 62, 210]. Innate immunity is essential for mediating both allograft rejection and early graft failure [230]. Both direct and indirect antigen presentation play a role in alloimmunity [78], and TLR activation by endogenous and exogenous danger signals on donor or recipient APCs could enhance alloimmunity. As TLR4 has a wide range of both endogenous and exogenous ligands [170, 231], this study began with determining the role of TLR4 in islet grafts transplanted into MHC-mismatched recipients, followed by examining the role of TLR4 within the transplant recipient. This study also examined effects of MyD88 and/or TRIF deficiency on islet allograft survival.

3.2 RESULTS

3.2.1 Lack of TLR4 does not impact islet allograft survival

To determine the role of TLR4 in islet allograft rejection, STZ-induced diabetic BALB/c recipients were transplanted with either 500 Tlr4−/− (n=15) or 500 C57BL/10 (n=6) donor islets under the left kidney capsule. Blood glucose levels were similar in both groups over the duration of the experiment (Figure 7A) and there was no significant difference in graft survival (Figure 7B). Days to graft failure for Tlr4−/− and C57BL/10 islet allografts were 23.6±1.5 and 25.2±1.6 days, respectively (Table 1, P=NS). To investigate the role of TLR4 in the recipient in islet allograft rejection, we transplanted islets from BALB/c donors into STZ-diabetic Tlr4−/− or WT recipients (Figure 7C). No significant difference in islet allograft
survival was observed between $Tlr4^{-/-}$ (n=6) and C57BL/10 (n=5) recipients (Table 1, $P=NS$). Upon graft failure (return to hyperglycemia), excised grafts were processed for histological analysis. Hematoxylin and eosin staining of $Tlr4^{-/-}$ or C57BL/10 islet allografts in BALB/c recipients showed extensive lymphocyte infiltration (Figure 8).

To confirm that $Tlr4^{-/-}$ islets lacked TLR4 expression, RNA was extracted from islets isolated from both $Tlr4^{-/-}$ and C57BL/10 animals. RT-PCR was performed and no amplified product was seen for TLR4 cDNA in $Tlr4^{-/-}$ islets, confirming that $Tlr4^{-/-}$ islets do not produce TLR4 (Figure 9A). To confirm that TLR4-deficient animals did indeed lack functional TLR4, we tested B220$^+$ splenocytes from $Tlr4^{-/-}$ and C57BL/10 mice for reactivity to a known TLR4 agonist, LPS [232]. FACS analysis revealed that $Tlr4^{-/-}$ B220$^+$ splenocytes do not respond to LPS, as demonstrated by a lack of increased expression of the costimulatory molecules CD40 and CD86 (Figure 9B). By contrast, B220$^+$ splenocytes from C57BL/10 mice increased CD40 and CD86 coexpression 11-fold when exposed to LPS, confirming TLR4-mediated up-regulation of costimulatory molecules.

3.2.2 Normal function of syngeneic islet transplants from $Tlr4^{-/-}$ donors

To ensure that the failure of islet allografts from $Tlr4^{-/-}$ donors cannot be ascribed to defects in $Tlr4^{-/-}$ islet function, we performed syngeneic transplants using $Tlr4^{-/-}$ and C57BL/10 islets into STZ-diabetic recipients (n=3 per genotype). Both $Tlr4^{-/-}$ and C57BL/10 islet grafts maintained normoglycemia in syngeneic recipients for the duration of the transplants (up to 10 weeks; Figure 10A). Removal of the graft-bearing kidneys from transplant recipients at 72 days post-transplant resulted in rapid return of hyperglycemia confirming that the grafts were responsible for maintenance of normoglycemia. Insulin and glucagon immunostaining of islet graft paraffin sections revealed healthy islet morphology (Figure 10B & C). Pancreas was removed upon euthanasia of hyperglycemic recipients to determine if endogenous islet re-growth occurred; insulin and glucagon staining revealed little to few beta-cells but sustained numbers of alpha-cells, indicating that glycemia was regulated by the islet graft and not endogenous islets (Figure 10D & E).
Figure 7: \textit{Tlr4}^{-/-} islet grafts are not protected from allograft rejection.

Blood glucose levels of BALB/c mice transplanted with \textit{Tlr4}^{-/-} or C57BL/10 islet grafts (A). Five hundred islets from \textit{Tlr4}^{-/-} (n=15) or C57BL/10 (n=6) donors (H-2B) were transplanted to the left kidney capsule of BALB/c recipients (H-2D) made diabetic by STZ administration 5 to 7 days prior to transplantation. All mice returned to normoglycemia within two days of transplantation and blood glucose levels were monitored every 2-3 days as a measure of graft function. Islet grafts from \textit{Tlr4}^{-/-} and C57BL/10 donors had similar allograft failure rates (B; 23.6±1.5 vs. 25.2±1.6 days to graft failure; \(P=\text{NS}\)). BALB/c islet grafts transplanted into STZ-\textit{Tlr4}^{-/-} or STZ-C57BL/6 recipients also had similar graft failure rates (C; 29.7±5.1 vs. 21.0±1.2 days to graft failure; \(P=\text{NS}\)).
Figure 8: Immune cell infiltration in Tlr4^{-/} and C57BL/10 islet allografts.

Hematoxylin and eosin-stained grafts excised from hyperglycemic BALB/c recipients reveal extensive immune infiltration in both Tlr4^{-/} (A) and C57BL/10 (B) islet allografts (scale bar: 50 µm). Kidney morphology appears normal with infiltration of lymphocytes into the peripheral islet allograft, evidenced by large clusters of mononuclear cells.
Figure 9: Islets and splenocytes from Tlr4⁻/⁻ animals do not express functional TLR4.
RT-PCR analysis of islets from Tlr4⁻/⁻ (n=2) and C57BL/10 (n=2) mice showing lack of TLR4 mRNA in islets from Tlr4⁻/⁻ mice (A). GAPDH was used as a loading control. Flow cytometry analysis of B220⁺ splenocytes isolated from Tlr4⁻/⁻ (n=2) and C57BL/10 (n=2) donors demonstrating increased expression of the costimulatory molecules CD40 and CD86 in C57BL/10 but not Tlr4⁻/⁻ splenocytes in response to the TLR4 ligand LPS (B).
Figure 10: Syngeneic transplants of Tlr4<sup>−/−</sup> islets function normally in STZ-diabetic recipients.

Transplanted islets (500) from Tlr4<sup>−/−</sup> (n=3) and C57BL/10 (n=3) donors both maintained normoglycemia in STZ-diabetic C57BL/10 recipients (A). All recipients of islet grafts returned to hyperglycemia following removal of the graft on day 71 post-transplant. C57BL/10 (B) and Tlr4<sup>−/−</sup> (C) syngeneic islet grafts exhibit strong insulin (green) and glucagon (red) positivity (scale bar: 100 µm; inset - scale bar: 25 µm). Pancreas from STZ-diabetic, C57BL/10 (D) and Tlr4<sup>−/−</sup> (E) islet transplant recipients show mostly glucagon-positive (red) alpha cells, and few insulin-producing (green) beta cells, confirming that blood glucose levels were normalized due to islet grafts and not endogenous islet regrowth (scale bar: 25 µm).
3.2.3 TLR signalling does not play a role in islet allograft survival

Given that TLR4-deficient islets were not protected from allograft rejection, we sought to determine whether there might be any role for TLR signalling in islet allograft rejection. We therefore transplanted islets from mice lacking MyD88 (MyD88<sup>−/−</sup>), TRIF (Trif<sup>−/−</sup>), the combined knockout (MyD88<sup>−/−</sup>/Trif<sup>−/−</sup>) or C57BL/6 control mice into STZ-diabetic BALB/c animals (Figure 11). Similar times to graft failure were observed in all groups regardless of the genotype of the islet donor (Table 5).

Table 5: Days to failure of islet allografts from donors lacking TLR4, MyD88 or TRIF.

<table>
<thead>
<tr>
<th></th>
<th>Islet Allograft Donors</th>
<th>Days to Graft Failure</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Tlr4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>23.6±1.5</td>
<td>0.25</td>
</tr>
<tr>
<td>6</td>
<td>C57BL/10</td>
<td>25.2±1.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;/Trif&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>25.0±1.0</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6</td>
<td>22.0±3.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>22.3±1.4</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>C57BL/6</td>
<td>18.3±3.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Trif&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>22.3±1.4</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>C57BL/6</td>
<td>23.8±2.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>BALB/c (Tlr4&lt;sup&gt;−/−&lt;/sup&gt; recipient)</td>
<td>29.7±5.1</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>BALB/c (C57BL/10 recipient)</td>
<td>21.0±1.2</td>
<td></td>
</tr>
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</table>
Figure 11: Lack of TLR signalling molecules MyD88 and TRIF does not prolong islet allograft survival.

Islets from (A) MyD88\(^{-/-}\)/Trif\(^{-/-}\) (n=4), (B) MyD88\(^{-/-}\) (n=4), (C) Trif\(^{-/-}\) (n=9) and C57BL/6 controls were transplanted into STZ-diabetic BALB/c mice. In all groups, islet allograft survival was not significantly different from the control group (P=NS).
3.3 DISCUSSION

3.3.1 Islet allograft rejection is independent of TLR signalling in mice

Using a full MHC mismatch islet allograft model, we sought to determine how lack of TLR signalling affects islet allograft rejection by transplanting islet grafts from either Tlr4−/−, MyD88−/−, Trif−/− or MyD88+/Trif−/− animals into STZ-diabetic recipients. We chose to specifically examine the role of TLR4 due to the wide array of exogenous and endogenous ligands and because it has been implicated as a potential mediator of allograft rejection in islet, liver, lung and kidney transplantation [203-205, 233]. Furthermore, many TLR4 endogenous ligands are released during tissue injury and transplantation, which may serve to exacerbate an allograft response. While TLR pathways have been previously implicated in mediating allograft rejection in minor antigen mismatch allograft models, absence of TLR4, MyD88 or TRIF in donor islets had no impact on the survival of MHC-mismatched mouse islet allografts. In addition, the absence of TLR4 in recipients of islet allografts made no significant difference to transplant outcomes.

The findings of this study are in agreement with a recent report by Zhang et al. examining the role of TLR4 in islet allograft rejection in both the recipient and donor [234]. Similar to our findings, using three separate strains of TLR4 deficient mice, this group observed that lack of TLR4 in donor islets did not impact islet allograft survival. This group also reported that a moderate, albeit significant, increase in islet allograft survival was observed with TLR4-deficient recipients. While we also see an increase in islet allograft survival, this difference was not significant, and Zhang et al. required the use of rapamycin to prolong graft survival in this model. Our findings and those by Zhang et al. corroborate a recent report that absence of TLR4 does not delay the rejection of MHC-mismatched skin allografts [206].

Other studies, however, have raised the possibility that TLR4 signalling may play a role in islet allograft rejection. In apparent contrast with our findings, Goldberg and colleagues reported prolonged survival of 4 out of 6 mouse islet allografts from TLR4 deficient donors [233]. Protection from allograft rejection was also conferred in 3 out of 4 recipients transplanted with islets infected with a TLR4-dominant negative adenovirus. One potential explanation for the differences between our findings and those reported by
Goldberg et al. may be the addition of polymyxin B by this group to islet culture medium, to deplete any contaminating LPS that might be present following islet isolation. To address this possibility, we performed additional transplant experiments with the addition of polymyxin B to media during overnight culture of Tlr4−/− islets prior to transplant. Even under these conditions, no improvement in islet allograft survival was observed. To determine whether defects in islet function could explain the failure of islet allografts in our hands, additional syngeneic transplant experiments were performed. Syngeneic grafts from Tlr4−/− and WT islet donors performed equally well, indicating that no obvious defects in islet function could explain the rapid failure of Tlr4−/− islet allografts.

Although our results and those of Zhang et al. speak against a critical role for TLR4 signalling in islet allograft rejection in donor tissue, TLR4 may still be important for regulating early graft function and immunological tolerance. HMGB1 is an endogenous ligand for TLR2 and TLR4, and is released by pancreatic islets upon transplantation [234, 235]. TLR4 mRNA is upregulated in islet allografts, as is HSP70, another endogenous TLR ligand [234]. Using a syngeneic suboptimal islet graft model (200 islets per transplant), Matsuoka et al. established that anti-HMGB1 antibody treatment can prevent early islet graft loss, and mice lacking known HMGB1 receptors TLR2 or RAGE are protected from suboptimal islet graft hyperglycemia [235]. Similarly, treatment with anti-IL-12 and anti-CD40L antibodies also improved hyperglycemia in this suboptimal syngeneic transplant model, indicating that preventing inflammation in response to transplantation can improve graft function. Matsuoka et al. also concluded that the site of transplantation was important in mediating inflammation as organs differ in endogenous inflammatory mediator populations [235]. This group reported that dendritic cells, natural killer (NK) T cells and neutrophils were important for mediating post-transplant inflammation in the liver. HMGB1 acts upon dendritic cells, which then activate NKT cells to produce IFN-γ, thereby stimulating neutrophils to also produce IFN-γ. Thus, early graft dysfunction and inflammation due to host TLR activation may be induced via the production of HMGB1 by donor islet grafts.

TLR signalling within the recipient may be essential for regulating immunological tolerance as well. Zhang et al. established long-term graft acceptance in Tlr4−/− recipients of MHC-mismatched islet allografts using a low-dose rapamycin protocol, and interestingly,
this group observed elevated levels of intragraft IL-10 and regulatory T cells [234]. \textit{Tlr4}$^{-/-}$ recipients also had significantly fewer macrophages within the graft and decreased IFN-\(\gamma\) mRNA. Depletion of CD25$^+$ cells at the time of transplantation with the administration of an anti-CD25 monoclonal antibody prevented long-term allograft survival in rapamycin treated \textit{Tlr4}$^{-/-}$ recipients, indicating that natural regulatory T cells play a role in maintaining graft tolerance in this model. Using \textit{in vitro} studies, this group also observed that TLR4 activation by LPS inhibited regulatory T cell development in the presence of TGF-\(\beta\), and increased IL-17. These findings demonstrate that TLRs play a role within the recipient to direct T-cell polarization and suppress the action of regulatory T cells to stimulate allograft rejection. Translating these findings to a clinical setting, suppression of TLR signalling in graft recipients using therapeutics could be an alternative way to promote graft survival by enhancing regulatory T cell function and immunological tolerance.

TLR4 signals via two downstream molecules, MyD88 and TRIF, which in turn activate NF-\(\kappa\)B and type 1 interferon production, respectively [169]. To assess the role of TLR downstream signalling molecules in islet allograft rejection, islet allografts were performed using islets from mice lacking MyD88, TRIF or both MyD88 and TRIF. Lack of either or both of these critical TLR signalling molecules in donor islets had no impact on islet allograft survival. These findings are in agreement with reports suggesting that MyD88 signalling does not affect survival of fetal porcine islet xenotransplants [236] and that blocking MyD88 signalling facilitates long-term acceptance of skin allografts only in the presence of costimulation blockade [186].

While these findings speak against an essential role for TLR4 and indeed any TLR signalling pathways in initiation of islet allograft rejection, it remains possible that under certain conditions and in less stringent allograft models that TLR signalling pathways may play a role. Indeed, MyD88-dependent TLR signalling has been reported to be involved in minor-antigen-mismatched allograft rejection, a less stringent allograft model [200]. Allograft rejection in this model could not occur in the absence of MyD88 and the proposed mechanism of protection was due to a decreased number of dendritic cells within the draining lymph nodes, reducing alloreactive T-cell activation. Lack of MyD88 was determined to decrease Th1 immunity, but leave Th2 immunity intact [200].
In a follow-up study by the same group, it was observed that MyD88 signalling was not critical for the rejection of MHC-mismatched skin and cardiac allografts, aligning with our islet transplant data [202]. While absence of MyD88 did not impact dendritic cell costimulatory molecule expression, or the ability of allogeneic APCs to stimulate alloprimed T cells, MyD88 signalling was observed to be essential for the dendritic cell maturation and priming of recipient T cells post-transplantation. TLR signalling therefore appears to play a role in supplementing the alloimmune response, but allograft rejection can occur independently of TLR function. These data also point to the potential role for TRIF in mediating dendritic cell function, since costimulatory molecule upregulation was not inhibited in the absence of MyD88.

Besides MyD88, TLR4 can also act via TRIF, the essential downstream molecule for TLR3 signalling. Although TLR3 activity has been implicated in viral induction of autoimmune diabetes, our studies indicate that lack of TLR3 signalling via TRIF is not essential for islet allograft rejection [159]. Although simultaneous absence of MyD88 and TRIF may delay major and minor histocompatibility antigen mismatch allograft rejection in the skin [201], it does not result in prolonged islet allograft survival, at least in our full MHC-mismatch allograft model.

Both the direct and indirect pathways of antigen presentation are thought to be important in activation of an allogeneic response [237-239]. Resident islet APCs may be largely responsible for direct antigen presentation and a subsequent alloimmune response in islet allotransplantation. Resident islet APCs are able to present beta-cell antigen to CD4+ T cells ex vivo and depletion of resident islet APCs can lead to islet allograft tolerance [240, 241]. Lack of TLR4 on donor APCs likely does not impact allograft rejection, as this study revealed no differences in allograft survival between Tlr4−/− and C57BL/10 islet donors; however, in vitro studies were not completed to determine whether defects existed in Tlr4−/− APCs. While direct antigen presentation is important for initiating an alloimmune response, host TLR signalling is essential for mediating early graft inflammation and suppressing regulatory T cell function. Therapeutically targeting TLR pathways within transplant recipients may improve transplant outcomes.

Although islet allograft rejection occurs independently of TLR signalling, other scavenger receptors may be important in the generation of an alloimmune response.
Monocytes engulf allogeneic endothelial cell membrane through a scavenger receptor dependent mechanism, which initiates monocyte T-cell stimulatory capacity, and the subsequent generation of an alloimmune response [242]. Also, macrophage type A scavenger receptor mRNA levels were reported to be elevated in rat cardiac allografts [243]. The mannose receptor and complement receptors are other examples of innate immune receptors that are present on monocytes and resting macrophages, and play a role in macrophage function [244]. The mannose receptor is capable of inhibiting Th1 responses by interfering with TLR/IL-1R signalling by upregulating expression of IL-1R type II, a decoy receptor, and secretion of IL-10 and IL-1Ra [245]. Polarization of macrophage (e.g. M1 vs. M2) and T-cell function dictates the nature of an inflammatory response; a shift from Th1 to Th2 type immunity corresponds to acute and chronic inflammation, and similarly, a M1 to M2 shift occurs during the transition from infection to wound healing [244]. In transplantation, TLRs work in coordination with other innate receptors to direct alloimmune responses and therefore, it is not surprising that deficiency of one TLR would not completely abrogate alloimmunity.

3.3.2 Future directions for TLRs in islet transplantation

Islet transplants are hindered by a myriad of immunological and non-immunological factors that affect graft viability. Chronic graft failure continues to limit the ability of type 1 diabetic islet transplant recipients to remain insulin injection free. Although the data reported in this study suggest that TLR signalling is not essential for islet allograft rejection, TLR signalling may still be an important contributor to the inflammatory milieu induced in transplantation. The current body of evidence suggests that TLR signalling in the recipient, but not the donor, plays a role in early graft failure and primary graft non-function. Performing suboptimal islet allotransplants using TLR-deficient recipients may be useful in determining the role of TLRs in early graft dysfunction and regulatory T cell function in alloimmunity. Also, as human islet transplants undergo different procedures and manipulations compared to experimental mouse islet transplants, it may be useful to determine the TLR expression profile of human islet grafts before and after transplant to obtain clues towards developing therapeutics. Understanding the role of TLRs on human islet graft function and survival could be tested in vivo using a “humanized” murine islet
allograft model; an immunodeficient mouse is equipped with human peripheral blood mononuclear cells and serves as the recipient of a human islet allograft [246]. Manipulation of TLR activation and expression in the recipient could help to elucidate the role of TLRs in human islet graft function and survival. Islet function in type 2 diabetes and islet transplantation share similar pathologies, such as an elevated proinsulin to insulin ratio and amyloid plaques, and targeting inflammatory pathways may decrease the proinsulin to insulin ratio in transplant patients as it has in patients with type 2 diabetes [150, 247]. TLR signalling may contribute to IAPP-induced beta-cell toxicity as TLR4 was observed to mediate neuronal apoptosis in response to amyloid beta-peptide [248]. Understanding how TLR pathways affect islet transplants in the immediate and long-term post-transplant period may be important to preventing acute and chronic graft failure. Maintaining graft function may not only extend the longevity of the graft, but also decrease the need for a top-up graft, and the requirement for multiple tissue donors. Inhibiting innate immunity in islet transplantation helps to prevent early graft failure, and combined with low-dose immunosuppression, can prevent long-term allograft rejection.

Immune suppression in islet transplantation requires multiple drug regimens. The identification of new targets might decrease the need for immune suppression. The immunosuppressive drugs tacrolimus, cyclosporine and azathioprine were recently observed to have no effect on in vivo IL-6 production in response to TLR agonists LPS and polyI:C, whereas glucocorticosteroids decreased IL-6 and IFN-α production [249]. As immunosuppressants primarily target T-cell proliferation and maturation, innate immunity likely remains active during immunosuppressive therapies, potentially contributing to graft dysfunction, increased islet apoptosis and eventual graft failure. Understanding the role of TLRs in graft failure in the presence of immunosuppressive drugs may provide new insight into the mechanisms behind chronic graft failure.

While the experimental models in this thesis examined allograft failure, islet transplants to TLR deficient-NOD mice would help to elucidate the effect of TLR pathways on both autoimmunity and alloimmunity. Dysregulated TLR4 expression has been associated with type 1 diabetes in the NOD mouse, and this could potentially relate to the ability of TLR activation to inhibit the maturation/development of regulatory T cells [188]. Also, MyD88 has been shown to be important in the development of autoimmunity in the
NOD mouse [193]. Further studies are clearly required to elucidate how TLR signalling impacts allograft rejection in recipients with type 1 diabetes.
CHAPTER 4: THE ROLE OF TLR SIGNALLING IN NORMAL
BETA-CELL FUNCTION & GLUCOSE HOMEOSTASIS

4.1 BACKGROUND

Type 2 diabetes is a multi-faceted disease, regarded as both a metabolic and an inflammatory syndrome. Establishing the inflammatory pathways that induce chronic inflammation in diabetes is essential to preventing disease progression and complications. While the loss of beta-cell mass and onset of hyperglycemia are orchestrated by multiple mechanisms, TLRs may play an essential role in initiating the destructive inflammatory cascade preceding these hallmark pathologies of diabetes. TLRs are present on a myriad of cell types including hematopoietic cells, endothelial cells and islet cells [156, 157, 159-161], all of which contribute to inflammatory action and potential islet dysfunction and apoptosis. Multiple groups have implicated TLRs in mediating FFA-induced insulin resistance [165, 218, 219, 250, 251], and TLR activation by endogenous danger signals could precipitate early beta-cell failure and hyperglycemia. While these data point to an important role for TLR signalling in contributing to disease pathogenesis, it remains to be determined how TLRs may contribute to glucose homeostasis under normal conditions. This study sought to determine the effects of TRIF and MyD88 deficiency on normal beta-cell function and glucose homeostasis. Given the pathogenic role TLRs play in promoting inflammation, inhibiting TLR activation under non-pathological conditions may serve to protect islets from inflammation-induced dysfunction and apoptosis. For this study, mice lacking the downstream TLR signalling molecules MyD88 or TRIF were used to encompass the effects of all TLR signalling pathways. While almost all TLRs use MyD88, TLR4 is unique in that it uses both MyD88 and TRIF. As TLR4 is widely implicated in diabetes-associated inflammation, both MyD88<sup>-/-</sup> and Trif<sup>-/-</sup> mice were studied.

4.2 RESULTS

4.2.1 Trif<sup>-/-</sup> mice are glucose intolerant

To determine how lack of TRIF signalling may impact glucose tolerance, we performed intra-peritoneal glucose tolerance tests (IPGTTs) on mice lacking TRIF, as well as
mice lacking TLR3 or TLR4. After a four-hour fast, Trif\(^{−/−}\) animals had significantly increased blood glucose levels compared to wild-type, age-matched controls (12.0±0.9 vs. 9.7±0.4 mM respectively; \(P<0.05\)). In Trif\(^{−/−}\) mice, blood glucose levels remained significantly higher than wild-type mice 60 (\(P<0.05\)) and 120 (\(P<0.005\)) minutes following glucose administration (Figure 12A). However, glucose tolerance in both Tlr3\(^{−/−}\) and Tlr4\(^{−/−}\) mice was similar to wild-type controls (Figure 12B & C). Interestingly, Tlr4\(^{−/−}\) mice had significantly lower blood glucose levels 30 minutes after glucose administration, indicating moderately improved glucose clearance. Therefore, a significant impairment in glucose tolerance was only apparent in mice lacking TRIF.

4.2.2 Trif\(^{−/−}\) and Tlr3\(^{−/−}\) mice are insulin sensitive

To understand the mechanism underlying the decreased glucose tolerance exhibited by Trif\(^{−/−}\) animals, we assessed insulin sensitivity by the insulin tolerance test (ITT). Despite significantly higher fasting blood glucose levels at the start of the test, Trif\(^{−/−}\) mice had similar insulin sensitivity compared to controls with significantly lower glucose levels 30 minutes after insulin administration (Figure 13A). Tlr3\(^{−/−}\) mice are also insulin sensitive (Figure 13B). Interestingly, Trif\(^{−/−}\) mice (n=10) had elevated fasting plasma insulin levels compared to wild-type controls (n=6) (180±22 vs. 89±24 pM; \(P<0.05\)), while Tlr3\(^{−/−}\) mice (n=5) had similar fasting plasma insulin levels to wild-type controls (122±17 vs. 89±24 pM; \(P=NS\)). Fasting plasma proinsulin levels in Trif\(^{−/−}\) (n=7) and wild-type (n=9) mice were similar (5.2±0.7 vs. 6.8±0.9 pM; \(P=NS\)). These findings suggest that despite high plasma insulin levels, Trif\(^{−/−}\) mice are insulin sensitive and therefore glucose intolerance in Trif\(^{−/−}\) mice is not likely to be due to reduced insulin sensitivity but rather changes in beta-cell mass or function.
Figure 12: Mice lacking TRIF, but not TLR3 or TLR4, are glucose intolerant.

Trif<sup>-/-</sup> (n=8; WT: n=10) mice have significantly impaired glucose tolerance compared to wild-type controls (A), whereas (B) Tlr3<sup>-/-</sup> (n=8; WT: n=8) and (C) Tlr4<sup>-/-</sup> mice (n=5; WT: n=8) have normal glucose tolerance. Tlr4<sup>-/-</sup> mice have slightly improved glucose clearance with significantly lower blood glucose levels 30 minutes post injection, compared to wild-type controls. Male, age-matched mice were fasted for four hours prior to the start of the IPGTT. Mice were injected with 50% dextrose and monitored over 120 minutes. Comparison of AUC values was used to determine significance (Trif<sup>-/-</sup> & WT IPGTT AUC values: 2850±236 vs. 2050±108; P<0.005). (**P<0.005, *P<0.05)
Figure 13: Lack of TRIF or TLR3 signalling in mice does not affect insulin sensitivity.

Male, Trif$^{/-}$ mice (n=9; WT n=7) and male, Tlr3$^{/-}$ mice (n=7; WT n=7) have similar insulin sensitivity to wild-type control mice (A, B). Trif$^{/-}$ mice have moderately albeit significantly improved blood glucose levels 30 minutes after insulin administration (*$P<0.05$). AUC values were used to determine significance ($P=NS$). All mice were fasted for four hours prior to the start of the insulin tolerance test and injected with 0.75 units of insulin per kilogram of body weight.
4.2.3 *Trif*<sup>−/−</sup> mice have increased beta-cell mass

To determine if the observed phenotype in mice lacking TRIF *in vivo* was associated with changes in beta-cell mass and/or a defect in beta-cell function, we next quantified beta-cell mass in *Trif*<sup>−/−</sup> and wild-type animals (Figure 14A). Surprisingly, beta-cell mass was markedly increased in *Trif*<sup>−/−</sup> animals (n=6) compared to wild-type (n=7) (3.5±0.9 vs. 1.7±0.2 mg; *P*<0.05). Immunostaining of *Trif*<sup>−/−</sup> islets with antibodies to insulin and glucagon showed that *Trif*<sup>−/−</sup> islets have normal islet architecture, with insulin-producing beta cells within the islet core and glucagon-producing alpha cells in the periphery (Figure 14B). Islet morphology in *Tlr3*<sup>−/−</sup> and *Tlr4*<sup>−/−</sup> mice also appeared normal (Figure 15). These data suggest that defects in beta-cell mass cannot explain the impairment in glucose tolerance in mice lacking TRIF and point to a defect in beta-cell function.

4.2.4 *Trif*<sup>−/−</sup> islets have impaired first-phase glucose-stimulated insulin secretion

We examined glucose-stimulated insulin secretion in static incubations of islets isolated from *Trif*<sup>−/−</sup> (n=3) and wild-type (n=3) mice (Figure 16A). At basal glucose levels (1.67 mM), *Trif*<sup>−/−</sup> islets had significantly lower insulin secretion than wild-type controls. When stimulated with high glucose (16.67 mM), *Trif*<sup>−/−</sup> islets responded with a three-fold increase in insulin secretion, whereas wild-type islets secreted eight-fold more insulin. Insulin content was not significantly different between groups (Figure 16B), whereas *Trif*<sup>−/−</sup> islet insulin secretion was significantly decreased in high glucose (88.6±4.1 vs. 380.3±25.9 ng/mL; *P*<0.05) (Figure 16C). These data suggest that beta cells lacking TRIF have a functional impairment in response to high glucose; differences at low glucose, although significant when normalized to insulin content, may be due to measurement error as insulin secretion was consistently similar between groups. In contrast, *Tlr3*<sup>−/−</sup> islets have normal function, as they have similar static glucose-stimulated insulin secretion compared to wild-type islets (Figure 16D).
Figure 14: \textit{Trif}^/- mice have increased beta-cell mass.

Male, 10-week old \textit{Trif}^/- mice (n=6) have significantly increased beta-cell mass compared to wild-type (n=7) controls (A; *P<0.05). Wild-type and \textit{Trif}^/- islets have similar morphology with strong insulin (red) and glucagon (green) immunostaining (B; scale bar: 100 µm).
Figure 15: *Tlr3*<sup>−/−</sup> and *Tlr4*<sup>−/−</sup> mice have similar islet morphology to wild-type controls. *Tlr3*<sup>−/−</sup> and *Tlr4*<sup>−/−</sup> islets have similar morphology to wild-type (WT), C57BL/6 islets with strong insulin (red) immunostaining of beta cells within the core of the islet, and glucagon (green) immunostaining of alpha cells on the periphery (scale bar: 25 µm).
Figure 16: Islets from mice lacking TRIF have impaired glucose-stimulated insulin secretion.

In a static incubation, $\text{Trif}^{-/-}$ islets secreted significantly less insulin compared to wild-type islets in a low and high glucose environment (A). Islets from $\text{Trif}^{-/-}$ (n=3) and wild-type (n=3) donors were incubated at low glucose (1.67 mM) for one hour prior to stimulation with high glucose (16.67 mM). $\text{Trif}^{-/-}$ and wild-type islets had similar insulin content at both low and high glucose (B), but significantly different insulin secretion at high glucose (C). Islets from $\text{Tlr3}^{-/-}$ (n=3) and wild-type (n=3) mice have similar glucose-stimulated insulin secretion in a static incubation (D). (**$P<0.005$, *$P<0.05$)
To understand further the defect in glucose-stimulated insulin secretion, we performed perifusion studies on isolated islets to assess dynamics of insulin secretion. Islets from \textit{Trif}^{-/-} (n=6) mice exhibited a reduced first-phase insulin response to glucose compared to wild-type (n=5) mice (Figure 17A); AUC values for the first-phase insulin response (0 to 20 minutes after glucose stimulation) from wild-type islets were significantly higher compared to \textit{Trif}^{-/-} islets (Figure 17B). By contrast, second phase glucose-stimulated insulin secretion remained intact. Insulin secretion in response to the non-glucose beta-cell secretagogues KCl and arginine was similar in wild-type and TRIF-deficient islets (Figure 17C). These findings suggest that the beta-cell exocytotic machinery required for insulin secretion is intact, and that the defect in glucose-stimulated insulin secretion in \textit{Trif}^{-/-} islets lies upstream of membrane depolarization, insulin granule docking and exocytosis. Together, these data raise the possibility that TRIF plays a previously unexpected role in glucose-stimulated insulin secretion in the beta cell.

4.2.5 \textit{Trif}^{-/-} and wild-type islets have similar GLUT2 localization

To determine whether defects in first-phase glucose-stimulated insulin secretion were related to changes in cellular GLUT2 localization, \textit{Trif}^{-/-} and wild-type pancreas sections were analyzed histologically for GLUT2. Immunofluorescent staining revealed that both \textit{Trif}^{-/-} and wild-type islets have strong GLUT2 and insulin positivity, with similar GLUT2 colocalization throughout the islet (Figure 18).
Figure 17: Islets from $Trif^{-/}$ mice have impaired glucose-induced first-phase insulin release in vitro.
Perifusion studies revealed that islets from $Trif^{-/}$ mice (n=6) have reduced first-phase insulin release but have similar second-phase insulin secretion compared to islets from wild-type mice (n=5) (A). AUC values comparing first-phase insulin release (0 to 20 minutes after glucose stimulation) show that wild-type islets secreted significantly more insulin than $Trif^{-/}$ islets (B). Insulin secretion in response to KCl and arginine was similar with $Trif^{-/}$ and wild-type islets. Basal levels of media contained 3 mM glucose (C). (*$P<0.05$)
Figure 18: *Trif*−/− mice have similar beta-cell GLUT2 localization.
Pancreata from male, 10-week old *Trif*−/− mice have similar GLUT2 localization compared to wild-type controls (scale bar: 100 µm).
4.2.6 Glucose intolerance and fasting hyperglycemia are moderately increased by high-fat diet in mice lacking TRIF

To establish whether mice lacking TRIF were more susceptible to high-fat diet-induced glucose intolerance, hyperglycemia and insulin resistance, 10-week old male $\text{Trif}^{-/-}$ (n=6) and wild-type (n=5) mice were placed on high-fat diet for 12 weeks. To assess glucose tolerance and insulin sensitivity, IPGTTs and ITTs were performed at 0 and 12 weeks. Blood samples were taken before the start of each test to examine blood glucose and fasting plasma insulin levels. After 12 weeks on diet, $\text{Trif}^{-/-}$ mice had significantly higher fasting blood glucose levels (14.6±2.1 vs. 10.3±0.8 mM; $p<0.05$) as well as significantly worse glucose tolerance (3540±212 vs. 2935±158 12 week IPGTT AUC; $p<0.05$) (Figure 19A & B). $\text{Trif}^{-/-}$ mice exhibited elevated body mass at the beginning of the study (27.2±0.5 vs. 22.8±1.3 g; $P<0.05$); however, after 12 weeks on diet, body mass was similar to high-fat fed wild-type mice (34.3±2.1 vs. 33.4±1.6 g; $P=\text{NS}$) (Figure 19C). Plasma insulin levels rose significantly after 12 weeks on diet in both $\text{Trif}^{-/-}$ mice (472±110 vs. 849±122 pg/mL; $P<0.05$) and wild-type mice (371±83 vs. 1324±285; $P<0.05$) (Figure 19D). Despite elevated plasma insulin levels, insulin sensitivity remained unchanged after 12 weeks on high-fat diet (Figure 19E).
Figure 19: High-fat diet moderately amplifies glucose intolerance and fasting hyperglycemia in Trif<sup>−/−</sup> mice.

Trif<sup>−/−</sup> (n=6) and wild-type (n=5) mice were given 45% kcal high-fat diet for 12 weeks and assessed for glucose and insulin tolerance, body mass, and fasting plasma insulin and glucose levels. Fasting hyperglycemia (A) and glucose intolerance (B) is significantly worse in Trif<sup>−/−</sup> mice compared to wild-type mice after 12 weeks on high-fat diet. Body mass (C) and plasma insulin (D) levels significantly increased in both groups after 12 weeks on high-fat diet. Insulin sensitivity (E) remained unchanged between groups over the 12 week high-fat diet. (**P< 0.005, *P<0.05)
4.2.7 Gene array analysis of Trif−/− and wild-type islets

To gain further insight into the mechanism behind the observed impairment in insulin secretion in Trif−/− islets, we performed genome-wide analysis to examine differential gene expression between wild-type (C57BL/6) and Trif−/− islets (n=4 mice per group) (Figure 20). Expression of detectable genes between the two groups had a high coefficient of correlation (r²=0.96); only genes with a signal significantly different from background were analyzed (detection p-value <0.05). The most significantly underexpressed and overexpressed genes are listed in Table 6; genes of interest with similar gene expression are also listed. Naip2, NLR family apoptosis inhibitory protein 2, was the most significantly downregulated gene in Trif−/− islets, while Arhgef19, Rho guanine exchange factor 19, was the most significantly upregulated gene. Gene expression of Trif (Ticam-1), MyD88 and Tirap, another TLR signalling molecule, was similar between groups. Real time RT-PCR was used to confirm Naip2, Ly96 and Arhgef19 gene expression analysis. A decrease in Naip2 mRNA expression was confirmed by real time RT-PCR (Figure 21A); however, no change in Ly96 or Arhgef19 mRNA expression was observed (Figure 21B & C). Further studies are required to determine whether changes in Naip2 mRNA are reflected in protein changes as well as establishing the effects of Naip2 deficiency on islet function.
Table 6: Comparative gene expression in $Trif^{-/-}$ and C57BL/6 (WT) mice.

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<th>WT Standard Deviation</th>
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<th>$Trif^{-/-}$ DiffScore*</th>
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* DiffScore = 10 log[P-value]
Figure 20: Differential gene expression analysis of $Trif^{-/-}$ and wild-type islets. 

Genome-wide analysis was performed using RNA isolated from $Trif^{-/-}$ and wild-type (C57BL/6) islets. Gene expression was compared between the two groups ($Trif^{-/-}$: Transgenic, y-axis; wild-type: x-axis); a high correlation coefficient was observed between groups ($r^2=0.96$). The two red outlier lines represent a 5 fold increase (above center line) or decrease (below center line) in gene expression compared to wild-type controls.
Figure 21: NAIP2 mRNA levels are significantly lower in islets isolated from Trif\textsuperscript{−/−} mice, while LY96 and ARHGEF19 levels are similar.

RNA isolated from Trif\textsuperscript{−/−} and wild-type islets was analyzed to confirm gene expression data determined by array. NAIP2 levels reflected the array results, with significantly lower levels in Trif\textsuperscript{−/−} islets (A), whereas LY96 (B) and ARHGEF19 (C) levels were similar. Gene expression was analyzed using the comparative threshold method and normalized to GAPDH levels. (** P<0.005)
4.2.8 Mice lacking MyD88 have normal glucose tolerance and insulin sensitivity

To determine whether lack of the TLR signalling molecule MyD88 affects glucose homeostasis and normal beta-cell function, we performed glucose and insulin tolerance tests on age-matched, male MyD88−/− mice and littermate controls, MyD88+/+. As shown in Figure 22, mice lacking MyD88 (n=9) have similar glucose tolerance (A) and insulin sensitivity (B) to their littermate controls (n=8). Fasting blood glucose levels (10.4±0.6 vs 9.4±0.8 mM; P=NS) were also similar. Interestingly, MyD88−/− (n=8) mice have elevated plasma insulin and proinsulin levels, while maintaining a similar proinsulin to insulin ratio compared to MyD88+/+ littermate controls (n=9) (Figure 23A, B &C). Plasma glucagon levels were not significantly different between MyD88−/− (n=4) and MyD88+/+ (n=4) mice (52.4±5.4 vs. 74.9±24.7 pg/mL; P=NS) (Figure 23D).

4.2.9 MyD88−/− mice have impaired glucose-stimulated insulin secretion

To further understand the role of MyD88 in beta-cell function, we examined glucose-stimulated insulin secretion in vitro. Despite normal glucose tolerance in vivo, islets from MyD88−/− mice (n=6) have impaired glucose-stimulated insulin secretion compared to littermate controls (n=6) in static incubations (Figure 24A). Insulin content, as a percentage of total protein content, was similar at both low (1.67 mM; 9.8±3.8 vs. 14.2±2.2 %) and high (16.7 mM; 7.3±4.2 vs. 12.8±1.8 %) glucose (Figure 24B). Insulin secretion in MyD88+/+ islets was significantly higher at high glucose (227.4±68.9 vs. 114.0±27.4 ng/mL; P<0.05), while insulin secretion was similar between groups at low glucose (Figure 24C).
Figure 22: Mice lacking MyD88 have normal glucose tolerance and insulin sensitivity. Age-matched, male $\text{MyD88}^{-/-}$ ($n=9$) and $\text{MyD88}^{+/+}$ ($n=8$) have similar glucose tolerance as revealed by IPGTT (A). Insulin sensitivity assessed by ITT (B) and fasting blood glucose levels (10.4±0.6 vs 9.4±0.8 mM; $P=\text{NS}$) were also similar between groups. All animals were fasted for four hours prior to testing.
Figure 23: MyD88−/− mice have elevated fasting plasma insulin and proinsulin levels.

MyD88−/− (n=8) mice have significantly elevated fasting plasma insulin (A) (274.3±55.5 vs. 132.0±19.1 pM; P<0.05) and proinsulin (B) (10.8±0.9 vs. 7.3±0.7 pM; P<0.05) levels compared to littermate controls (n=9). Proinsulin (PI) to insulin (I) ratios in MyD88−/− mice were similar to littermate controls (C; 5.4±0.6 vs. 5.5±1.1 %; P=NS). MyD88−/− plasma glucagon levels (52.4±5.4 vs. 74.9±24.7 pg/mL; n=4 per group; P=NS) were not significantly different from MyD88+/+ littermate controls (C). (* P<0.05)
Figure 24: MyD88−/− mouse islets have impaired glucose-stimulated insulin secretion in static cultures.

Insulin secretion as a percent of insulin content (A) from MyD88−/− islets was significantly lower at high glucose compared to littermate controls (0.42±0.12 vs. 0.22±0.05 %; \( P<0.05 \)). This defect was primarily due to a secretory defect as islet insulin content was similar in both conditions (B). Insulin secretion at high glucose (C) was significantly higher with MyD88+/+ islets (227.3±68.9 vs. 114.0±27.4 ng/mL; \( P<0.05 \)). (* \( P<0.05 \))
To establish the dynamics of insulin secretion in \(MyD88^{-/-}\) and \(MyD88^{+/-}\) islets, we performed islet perifusion studies. Interestingly, insulin secretion in response to glucose was similar between genotypes with a moderate first-phase peak, followed by a sustained second-phase (Figure 25A); area-under-the-curve (AUC) values (Figure 25B) for first phase insulin secretion (0-20 minutes after glucose infusion) were compared to determine significance (62.3±2.7 vs. 63.3±11.9; \(P=NS\); n=3 animals per group). Insulin secretion in response to non-glucose secretagogues KCl and arginine was similar between groups (Figure 25C), demonstrating that beta-cell exocytotic machinery required for insulin secretion was intact. These results point to a potential defect in collective insulin secretion over time, as in the static cultures, insulin secretion is lower in \(MyD88^{-/-}\) islets after 1 hour at high glucose. Due to the sustained response in insulin secretion with KCl and arginine, we suspect the defect in insulin secretion in \(MyD88^{-/-}\) islets lies upstream of membrane depolarization, insulin granule docking and exocytosis and instead may relate to a feedback or regulatory defect within the beta-cell.

4.2.10 \(MyD88^{-/-}\) mice have increased beta-cell replication but similar beta-cell mass

To establish whether elevated plasma insulin and proinsulin levels and impaired glucose stimulated insulin secretion were related to differences in beta-cell morphology, beta-cell replication and mass were measured (Figure 26). BrdU incorporation revealed that \(MyD88^{-/-}\) animals (n=7) had a significantly increased number of replicating beta cells compared to littermate controls (0.058±0.004 vs. 0.043±0.005 (n=4) ratio of BrdU/insulin double-positive cells to total number of insulin-positive cells per islet; \(P<0.05\)) (Figure 26A). Interestingly, however, \(MyD88^{-/-}\) (n=7) and \(MyD88^{+/-}\) (n=6) animals had similar beta-cell mass (1.53±0.18 vs. 1.43±0.16 mg; \(P=NS\)) (Figure 26B). We also compared beta-cell size by calculating the ratio of the number of insulin-positive cells to insulin-positive islet area, and similar to beta-cell mass, there was no difference between genotypes (0.0072 ± 0.0004 vs. 0.0073 ± 0.0006; \(P=NS\)) (Figure 26C). Insulin and glucagon immunostaining revealed similar islet architecture with alpha cells on the perimeter and beta cells within the islet core (Figure 26D). These results point to a potential role for MyD88 in beta-cell replication or turnover.
Figure 25: Dynamic insulin secretion as assessed by perifusion is similar with both MyD88<sup>-/-</sup> and MyD88<sup>+/+</sup> islets.

Perifusion studies reveal no differences in first or second phase insulin secretion in response to glucose (A). First-phase AUC values (B) were similar between MyD88<sup>-/-</sup> and MyD88<sup>+/+</sup> islets. Insulin response to non-glucose secretagogues KCl and arginine was also similar between groups (C).
Figure 26: *MyD88*⁻/⁻ mice exhibit increased beta-cell replication and similar beta-cell mass. *MyD88*⁻/⁻ mice have significantly elevated beta-cell replication as determined by BrdU incorporation (A). Beta-cell mass was similar between groups (B). Beta-cell size was compared using the ratio of beta-cell nuclei to insulin positive area and no difference was found between groups (C). Insulin (red) and glucagon (green) staining reveal normal islet architecture with an alpha-cell mantle and beta-cell core (scale bar: 25 µm) (D). (* P<0.05)
4.2.11 *MyD88*⁻/⁻ and *MyD88*⁺/⁺ mice have similar beta-cell morphology and polarity

To determine whether defects in insulin secretion and content were related to changes in expression of essential beta-cell proteins, we analyzed pancreas from *MyD88*⁻/⁻ and *MyD88*⁺/⁺ animals for GLUT2 and PDX-1 expression. As a necessary glucose transporter for beta cells, changes in GLUT2 expression could explain differences in *in vitro* glucose stimulated insulin secretion not seen *in vivo*. GLUT2 expression was similar in *MyD88*⁻/⁻ islets compared to littermate controls with cytoplasmic and membrane bound immunopositivity (Figure 27A). Real time RT-PCR analysis of *MyD88*⁻/⁻ and *MyD88*⁺/⁺ islets for GLUT2 mRNA expression revealed similar levels between groups (Figure 27B). Immunostaining for PDX-1 revealed strong positivity in insulin-expressing beta cells (Figure 28).

As differences in beta-cell polarity have been shown to affect beta-cell function [252, 253], we sought to determine whether beta-cell polarity was affected in the absence of MyD88. Nuclei from *MyD88*⁻/⁻ beta cells appear clustered towards vascular endothelial cells, such that the nuclei lie closer to the basal membrane compared to the littermate controls (Figure 29A). When quantified, however, there was no difference between genotypes in the relative distance between beta-cell nuclei and the rosette center (Figure 29B). Quantification was based on the geometric center of the rosette; however, not all rosettes were represented by a planar section, allowing for some measurement error. Therefore, while the quantification method was capable of highlighting major discrepancies in nucleus distance between genotypes, it may not be sensitive to subtle differences. These observations suggest that beta-cell polarity is not significantly different between genotypes and the impairment in insulin secretion in *MyD88*⁻/⁻ islets is not related to significant morphological changes.
Figure 27: *MyD88<sup>−/−</sup>* and *MyD88<sup>+/+</sup>* mouse pancreas exhibit similar GLUT2 immunofluorescence.
Pancreata from *MyD88<sup>−/−</sup>* (n=6) and *MyD88<sup>+/+</sup>* (n=6) animals were analyzed for GLUT2 expression, and both groups revealed strong GLUT2 (green) positivity within the membrane (A). An E-cadherin antibody (red) was used to verify membrane co-localization (scale bar: 25 µm). GLUT2 mRNA expression is similar in islets from *MyD88<sup>−/−</sup>* and *MyD88<sup>+/+</sup>* as measured by real-time RT-PCR (B).
Figure 28: Mice lacking MyD88 have strong PDX-1 expression within pancreatic beta cells. PDX-1 expression was analyzed in pancreata from $\text{MyD88}^{-/-}$ (n=6) and $\text{MyD88}^{+/+}$ (n=6) animals, and both groups show similar expression. PDX-1 nuclear positivity (green) correlated with strong insulin expression (red) (scale bar: 25 µm).
**Figure 29: Beta-cell polarity in *MyD88<sup>-/-</sup>* and *MyD88<sup>+/+</sup>* mice.**

Membrane (E-cadherin; green), vascular endothelium (MECA-32; red) and nuclear (DAPI; blue) staining of *MyD88<sup>-/-</sup>* and *MyD88<sup>+/+</sup>* pancreas revealed clustering of nuclei (arrows) in *MyD88<sup>-/-</sup>* beta-cells towards the basal membrane (A; scale bar: 25 µm). When quantified by measuring the relative distance of the nuclei to the center of the rosette (normalized to the distance between the apical membrane to center of rosette), no significant difference was found (B) (n=4 mice per genotype); however, some clustering appeared to be present.
4.2.12 Mice lacking both MyD88 and TRIF have slightly impaired glucose tolerance and normal insulin sensitivity compared to wild-type, C57BL/6 controls.

To determine whether the combined deficiency of MyD88 and TRIF affects glucose homeostasis in mice, glucose and insulin tolerance tests were performed on age-matched, male $^{MyD88^{-/-}/Trif^{-/-}}$ mice and wild-type controls (Figure 30). All mice were fasted for four hours prior to the start of metabolic testing. $^{MyD88^{-/-}/Trif^{-/-}}$ mice ($n=4$) have similar fasting plasma blood glucose levels ($12.3\pm1.0$ vs. $10.9\pm0.2$ mM; $P=\text{NS}$) and glucose tolerance to wild-type controls ($n=3$) (IPGTT AUC values: $2775\pm251$ vs. $2251\pm181$; $P=\text{NS}$) (Figure 30A). Interestingly, glucose levels remained higher than wild-type controls after 60 minutes and were significantly elevated in $^{MyD88^{-/-}/Trif^{-/-}}$ animals compared to wild-type controls at 120 minutes, mimicking glucose tolerance observed in $^{Trif^{-/-}}$ animals. Insulin sensitivity was similar between $^{MyD88^{-/-}/Trif^{-/-}}$ and wild-type mice (ITT AUC values: $33\pm1$ vs. $32\pm2$; $P=\text{NS}$), indicating that the phenotype seen in these mice may relate to the defects observed in $^{Trif^{-/-}}$ mice (Figure 30B). This is not surprising given that no differences in glucose or insulin tolerance were observed in $^{MyD88^{-/-}}$ mice. Further studies are required to determine whether $^{MyD88^{-/-}/Trif^{-/-}}$ mice possess impaired glucose-stimulated insulin secretion \textit{in vitro}, as observed with $^{Trif^{-/-}}$ mouse islets.
Figure 30: Mice lacking both MyD88 and TRIF have slightly impaired glucose tolerance and normal insulin sensitivity.

Age-matched, male MyD88+/Trif+/ mice (n=4) and wild-type, C57BL/6 controls (n=3) have similar fasting blood glucose levels and glucose tolerance. While glucose levels are significantly elevated at 120 minutes in MyD88+/Trif+/ mice, IPGTT AUC values were not significantly different. (* P<0.05)
4.3 DISCUSSION

4.3.1 TRIF is important for glucose homeostasis and normal beta-cell function

Inhibition of TLR activation has been previously shown to improve insulin resistance and prevent diet-induced obesity [165, 219, 251]. TLR2 and TLR4 have been implicated in mediating beta-cell toxicity due to the activation of these receptors by saturated free-fatty acids, which are present at high levels in obesity and type 2 diabetes. In order to understand the role of TLR signalling in normal islet function, mice lacking the TLR downstream signalling molecules TRIF or MyD88 were examined. TRIF is an essential signalling molecule for TLR4 and TLR3, and while it can directly bind to TLR3, it must use TRAM to bind to TLR4 [169]. Interestingly, we observed that TRIF deficiency induces decreased glucose tolerance and beta-cell dysfunction. Contrary to reports that TLR signalling is only detrimental to islet function, our findings suggest that TLR signalling via TRIF is required for normal beta-cell function and glucose tolerance. This finding is seemingly counterintuitive as lack of TLR signalling and the subsequent prevention of inflammation might be predicted to have a positive effect on beta-cell homeostasis. Although TLR pathways are known to be important sensors of invading pathogens or danger signals, these data indicate that an additional role for TLR signalling molecules exists in regulation of beta-cell mass and function. These findings share similarities to those in a recent study by Bollyky et al. who observed that lack of MyD88 increased susceptibility to STZ-induced apoptosis and caused a decrease in beta-cell mass [222]. We report here that lack of TLR signalling molecule TRIF is similarly detrimental to the beta-cell. Taken together, these data point to an important role for TLR signalling in the maintenance of beta-cell mass and function.

Because we studied mice with global TRIF deficiency and not mice with tissue specific deletion of TRIF, we cannot ascertain the critical tissues in which TLR-signalling deficiency leads to impaired glucose homeostasis. We observe beta-cell dysfunction and glucose intolerance in the absence of insulin resistance; however, further studies will be needed to elucidate the effect of TRIF deficiency on other sites of glucose homeostasis such as the liver and adipose tissue. Static incubation of isolated islets from mice lacking TRIF revealed a marked defect in glucose-stimulated insulin secretion. Subsequent perifusion
experiments using other secretagogues, to examine the dynamics of insulin secretion, confirmed the presence of this defect and moreover showed that it is characterized by a specific loss of first-phase insulin secretion in response to glucose. In this regard, the beta-cell defect in mice lacking TRIF resembles that seen in patients with type 2 diabetes, who typically also have impaired first phase glucose-stimulated insulin secretion [254]. The impaired insulin secretion could not be attributed to a loss of beta-cell mass. In the absence of TRIF, islet morphology appeared normal and beta-cell mass was increased, not decreased, perhaps as partial compensation for the insulin secretory defect.

Unlike TRIF-deficient animals, mice lacking TLR4 or TLR3 had normal glucose tolerance, even though these TLRs signal via TRIF. Although TLR4 could still signal via MyD88 in the absence of TRIF, TLR3 signalling is thought to be completely TRIF-dependent, and as a result lack of TLR3 would be expected to induce the same effects as lack of TRIF. We found, however, that this is not the case, as lack of TRIF, but not TLR3 or TLR4, induces beta-cell dysfunction and glucose intolerance. One possible interpretation of these data is that other TLRs can compensate for the deficiency in TLR3 or TLR4 signalling, and therefore events downstream of TRIF signalling can still occur even in the absence of TLR3 or TLR4. However, as this study and the recent report by Bollyky et al. demonstrate, when TLR signalling molecules are functionally impaired, beta-cell function or survival can be affected [222].

The high-fat diet study revealed that Trif−/− mice respond to an increase in dietary fat with a further decrease in glucose tolerance and worsening hyperglycemia compared to wild-type controls. While wild-type control mice exhibit a slight deterioration of glucose tolerance after 12 weeks on a high-fat diet, fasting blood glucose levels were not significantly different from baseline values. Trif−/− mice however exhibited increased fasting hyperglycemia and glucose intolerance, indicating a lack of compensation for increased dietary fat. Increases in body mass and plasma insulin levels in Trif−/− mice paralleled those seen in wild-type mice. This suggests that Trif−/− mice attempt to compensate for increased hyperglycemia and adiposity with an increase in plasma insulin. Interestingly, insulin sensitivity remained intact in both groups throughout the study. These findings raise the possibility that TRIF may play a role in beta-cell adaptation to dietary changes.
Gene expression analysis of islets isolated from mice lacking TRIF provided some intriguing results. Although there were no differences in gene expression of essential beta-cell function and survival genes such as Pdx1 and Glut2, changes in Naip2, Arhgef19, Ly96 or Slc39a10 may provide new information on the regulation of beta-cell function. NAIP2 belongs to a family of cytosolic pattern recognition receptors called NOD (nucleotide oligomerization domain) like receptors (NLRs) and contain a BIR (baculovirus inhibitor of apoptosis repeat) and LRR (leucine rich repeat) domain, similar to TLRs [255]. NLR family members and other proteins comprise intracellular inflammasomes responsible for responding to intracellular microbial pathogens [255]. Inflammasomes have been implicated in contributing to silicosis, asbestosis and Alzheimer’s disease [255]. The NLRP3/NALP3 inflammasome is important for IL-1β processing as it activates caspase-1, an essential enzyme for the conversion of pro-IL-1β. 

Naip2 has been described as a candidate gene in murine Legionella resistance [256]. TRIF action or function of downstream molecules may be important in regulating Naip2 gene expression. This finding may also represent an alternative role for NAIP2 in mediating glucose metabolism or glucose-stimulated insulin secretion.

The Slc39a10 gene encodes zinc transporter 10 (Zip10), which is important for zinc homeostasis in erythrocytes [257]. Interestingly, another zinc transporter gene SLC30A8 has been implicated as a candidate risk gene for type 2 diabetes and is expressed in beta-cells [92, 96, 258]. Deletion of the Slc30A8 gene in mice results in reduced glucose-stimulated insulin secretion and this was also associated with significantly lower islet zinc content and decreased plasma insulin levels [259]. Decreased first phase insulin secretion may relate to disturbed zinc homeostasis as zinc is essential for beta-cell insulin crystallization [260]. Although we did not see a difference in insulin content in Trif−/− islets, alterations in granule structure and insulin crystallization may exist.

The gene expression of Ly96 and Arhgef19 was determined to be significantly higher in Trif−/− islets by genome wide analysis; however, this finding was not confirmed by quantitative RT-PCR. Although mRNA expression of Arhgef19 is not significantly (P=0.07) upregulated in Trif−/− islets, it may still contribute to the exhibited phenotype outlined in Trif−/− mice. ARHGEF19 or WGEF is a guanine exchange factor encoded by Arhgef19 that is responsible for catalyzing the release of GDP on Rho family GTPases in exchange for GTP.
Rho family GTPases are involved in regulation of cytoskeletal organization, gene expression and membrane traffic [261]. Forced expression of WGEF in HeLa cells alters cell morphology, inducing membrane ruffling and formation of filopodia [261]. WGEF expression has been observed in intestine, liver, heart, kidney and adipocytes [261, 262]. WGEF expression in adipocytes was reported to decline during adipocyte maturation, and overexpression of WGEF down-regulated the adipocyte marker genes PPAR-γ and adipsin [262]. Mice with high fat diet-induced obesity exhibit lower expression of Wgef in white adipose tissue [262]. These results imply that WGEF expression may be important for keeping cells in an immature, plastic state, and WGEF may be involved in cellular adaptability to changes in energy consumption and metabolism. As WGEF is potentially increased in Trif−/− islets, poor glucose-stimulated insulin secretion may be related to compromised maturity of Trif−/− beta cells.

Trif−/− mice were created using N-ethyl-N-nitrosurea (ENU) induced germline mutagenesis, and possess an altered TRIF protein due to a single base pair deletion, resulting in an abolished response to TLR3 agonist and a dampened response to TLR4 agonist [263]. As the genetic defect in these mice was created in a non-specific manner, it is possible that other mutations have not been identified in this mouse, and could be contributing to this phenotype. Although the colony of Trif−/− mice was characterized by the manufacturer and bred through many generations both by the manufacturer and at the CFRI Animal Care Facility, the possibility exists that (ENU)-induced base pair mutations could still exist elsewhere in the genome.

Human TRIF gene expression is primarily regulated by NF-kB [264]. TLR2, TLR3 and TLR4 activity as well as TNF-α and IL-1α induce Trif expression in a NF-κB dependent manner. Type I and II interferons were observed to have no effect on Trif gene expression [264]. NF-κB is an important regulator of beta-cell survival and TRIF activation of NF-κB may be essential for other cellular functions like glucose-stimulated insulin secretion, zinc transport, and cytoskeletal organization [265, 266]. Multiple molecules downstream of TRIF are known to play established roles not only in inflammatory signalling, but also in replication and cell survival, as well as cell function. TLR signalling has been implicated in regulating cell cycle entry by targeting the cell cycle inhibitor p27 [267, 268]. TRIF interacts with TBK-1 (TANK binding kinase-1), a kinase essential in the activation of IRF-3 (IFN
regulatory factor 3) and subsequent production of IFN-β, and TBK-1 has been reported to regulate p27 expression, an established regulator of beta-cell proliferation [267, 269, 270]. IRF-3 is capable of activating NF-κB, which in turn can play a pro- or anti-inflammatory role within the islet [265, 266]. In addition, TLR3 activation of TRIF has been shown to require activation of the PI3K/Akt pathway to induce gene expression [271]. Since Akt has been shown to be an important regulator of beta-cell function, one possible mechanism is that TRIF deficiency leads to beta-cell dysfunction via decreased Akt signaling [272]. Recently, CXCL10 activation of TLR4 was shown to lead to beta-cell death by switching Akt signals from proliferation to apoptosis [160]. Since beta-cell mass was increased in TRIF-deficient animals, and Akt activation is associated with beta-cell proliferation, it seems unlikely that loss of Akt signalling in TRIF-deficient animals could explain the changes in beta-cell mass, but it is possible that this pathway underlies loss of glucose-sensing pathways, leading to beta-cell dysfunction. Double-stranded RNA-dependent protein kinase (PKR) is an intracellular pathogen sensor and was recently reported to coordinate nutrient, ER stress and pathogenic signals for regulation of insulin action and metabolism [273]. Although the PKR pathway acts independently of the TLR3 signalling pathway, perhaps TRIF is involved in the mutual activation of NF-κB and the proposed “metabolic inflammasome”, a node of inflammatory and insulin signalling [273]. Understanding the molecular mechanism underlying the impaired glucose-stimulated insulin secretion and increased beta-cell mass in TRIF-deficient mice will require further study.

Despite glucose intolerance, Trif−/− mice are insulin sensitive. This seems somewhat surprising given that plasma insulin levels in Trif−/− mice are significantly higher than wild-type controls, and elevated fasting insulin levels are a marker of insulin resistance. It is possible that differences in insulin sensitivity exist but could not be detected due to the limited sensitivity of the insulin tolerance test. Euglycemic clamp studies might reveal differences in insulin sensitivity between genotypes. Interestingly, Tlr4−/− mice are protected from diet-induced insulin resistance [165]. This mechanism of protection potentially centers around the ability of saturated free fatty-acids to activate TLR4 signalling and may not involve TRIF signalling, as TRIF only represents the latent, second phase of TLR4 signalling [156].
One limitation of this study is the use of wild-type, C57BL/6 controls in this study. Control animals were purchased from Jackson Laboratories, and metabolic testing commenced after a 7-day facility acclimatization period. However, differences in breeding and diet protocols between animal facilities may have impacted our study. Wild-type controls used in the high-fat diet study exhibited significantly lower baseline body mass levels, which could have related to differences in breeding and maintenance protocols, as well as metabolic changes during shipment. While C57BL/6 mice are the appropriate background control strain, littermate controls are regarded as superior due to similar breeding and housing conditions. Genetic drift within strains is another reason to avoid the use of a separate control strain, and another added benefit to the use of littermate controls.

4.3.2 The role of MyD88 in normal beta-cell function and glucose homeostasis

MyD88 is an essential downstream signalling molecule for all TLRs except TLR3, and for the IL-1R. Upon receptor activation, MyD88 recruits IRAK and activates NF-κB [169]. MyD88 is rapidly induced in response to IL-6 stimulation and is essential for IL-1 and IL-18 action [274]. Mice lacking MyD88 have normal glucose tolerance and insulin sensitivity, despite elevated plasma insulin and proinsulin levels. This is surprising given that elevated insulin levels are indicative of insulin resistance. However, IL-1R1 knockout animals similarly have increased basal insulin levels in the absence of hypoglycemia [136]. Despite elevated insulin and proinsulin levels, the ratio of proinsulin to insulin was similar between genotypes, indicating intact proinsulin processing within \( MyD88^{-/-} \) beta-cells. Although insulin tolerance, as measured through insulin tolerance test, revealed no differences between genotypes, euglycemic clamp studies may provide clues towards subtle changes in insulin sensitivity.

Static cultures of islets revealed that glucose-stimulated insulin secretion at high glucose was lower with \( MyD88^{-/-} \) islets. Given that insulin accumulation over a one-hour period could occur at various rates between genotypes, islet perifusion was performed to examine the dynamics of insulin secretion, and surprisingly, no difference was seen between \( MyD88^{-/-} \) and \( MyD88^{+/+} \) islets. Both sets of islets were analyzed after overnight culture; however, islet numbers were different between experiments; 20 islets in static cultures vs. 100 islets in perifusion columns were analyzed. Paracrine effects are present in static
cultures, but not in perifusion, and this could potentially explain the differences seen in insulin secretion. Collection of insulin or somatostatin within the well during static cultures may inhibit beta-cell secretion, whereas, during perifusion, such accumulation of islet hormones would not be expected to occur. In any case, beta-cell function may be largely overshadowed in vivo in MyD88<sup>−/−</sup> and MyD88<sup>+/+</sup> mice due to differences in hepatic glucose production and other regulators of glucose homeostasis, which would not be detected by insulin tolerance.

Beta-cell mass was not affected in mice lacking MyD88; however, beta-cell replication was significantly increased. As measurements were made simultaneously in the same mice, it is possible that increases in beta-cell proliferation have not yet developed into significant increases in beta-cell mass. The finding of impaired glucose-stimulated insulin secretion in vitro combined with increased beta-cell proliferation suggests a potential defect in beta-cell maturation or growth. We examined GLUT2 and PDX-1 expression with RT-PCR and immunofluorescence and found no difference in expression between genotypes. We also examined beta-cell polarity by measuring the distance between beta-cell nuclei and the center of beta-cell rosettes. Beta cells form rosettes around vascular endothelium as they are not capable of forming a basement membrane and changes in beta-cell polarity can affect beta-cell function [253, 275, 276]. Quantification did not reveal a significant difference in beta-cell polarity; however, in a variety of sections, some beta-cell nuclei clustering towards the basal membrane was observed.

While MyD88 is essential for TLR signalling, it also plays a significant role in mediating IL-1 action. Leptin is a glucose-regulatory hormone that acts on hypothalamic regions of the brain to decrease appetite, suppress insulin secretion and increase energy expenditure [137]. Leptin action in the hypothalamus is mediated by IL-1 action and the type 1 IL-1 receptor (IL-1R1) is expressed in the hypothalamus [138, 277, 278]. IL-1R<sup>−/−</sup> mice are resistant to the effects of leptin, and IL-1β is significantly upregulated in the hypothalamus upon injection of leptin in mice [138]. Because we are using a global knockout in this study, the potential role of MyD88-deficiency in the hypothalamus in our study must also be considered. Although no differences in body mass were seen in our cohort of mice, we did not examine food intake or body temperature, which may be affected by lack of MyD88. Within the islet, leptin acts to inhibit insulin secretion [137]. As IL-1R1
has been observed to be important for leptin signalling in the hypothalamus [138], IL-1R1 signalling may also be important for mediating leptin action in the beta cell. *MyD88*−/− mice may have mild leptin resistance due to impaired IL-1R1 activity and decreased leptin action may be responsible for increased fasting plasma insulin levels.

Similar to our observations in *MyD88*−/− mice, aged *Il-1r1*−/− mice exhibit basal hyperinsulinemia in the absence of hypoglycemia [136]. *Il-1r1*−/− mice also develop mature-onset obesity and become glucose intolerant and insulin resistant with age (20-24 weeks) [136]. Our study examined mice aged 8-12 weeks, and perhaps with increased age, differences in glucose homeostasis and insulin sensitivity could be observed. IL-1 has also been implicated in lipid accumulation, as IL-1 receptor antagonist (IL-1Ra) knockout mice are lean and resistant to diet induced obesity due to reduced total lipase activity. Low levels of IL-1β have been previously shown to positively influence beta-cell function and turnover via the Fas-FLIP pathway, and mice lacking IL-1β have decreased glucose-stimulated insulin secretion [145]. Collectively, these findings suggest that MyD88 signalling is beneficial to beta-cell function under normal conditions, whereas during inflammation, excessive MyD88 action can induce beta-cell dysfunction.

Our findings are in partial agreement with those recently reported by Bollyky et al. [222]. *MyD88*−/− mouse islets were reported to have increased apoptosis to multiple low-dose STZ. This correlates with our finding that beta-cells in *MyD88*−/− have a higher rate of replication as replicating beta-cells are more susceptible to apoptosis [279]. This increase in apoptosis susceptibility may also explain the similarity in beta-cell mass seen between genotypes in our study. One important difference between the Bollyky study and the current study is the use of littermate controls versus the use of purchased wild-type (C57BL/6) mice as controls in the Bollyky study. This difference may explain the discrepancies between studies in beta-cell mass as mice develop with different diets and conditions. All littermate controls in the current study were exposed to the same cage conditions, diet, drinking water and antibiotic treatment as the *MyD88*−/− mice. The use of antibiotics to maintain the *MyD88*−/− colony was not commented on in the Bollyky report and may represent an important difference between the control strain as the gut flora is an important factor in the susceptibility to diabetes [196].
Islets from mice lacking MyD88 secreted significantly less chemokine KC and IL-6 in response to glucose and palmitate, indicating a protective mechanism in glucolipotoxic environment [224]. While lack of MyD88 may be beneficial in inflammation and diet-induced obesity, in a baseline or normal environment, signalling through MyD88 via the IL-1 receptor or TLRs may be important for maintaining normal beta-cell function. Further studies are needed to determine the role for MyD88 in diet-induced obesity and diabetes; however, due to the expression of the IL-1R1 in the hypothalamus, tissue-specific knockout models are required.

4.3.3 Impact of MyD88 and TRIF deficiency on glucose tolerance and insulin sensitivity

Mice lacking both MyD88 and TRIF share similarities in glucose tolerance to Trif−/− mice. After 60 minutes post glucose injection, blood glucose levels remain high and are significantly higher at 120 minutes, pointing to a defect in glucose clearance, similar to Trif−/− mice. This difference in glucose tolerance could not be attributed to insulin resistance as insulin sensitivity was similar between MyD88−/−/Trif−/− and wild-type, C57BL/6 controls. This is perhaps not surprising as no differences in glucose tolerance or insulin sensitivity were observed in mice lacking MyD88. While we have not tested in vitro glucose-stimulated insulin secretion, one could predict that the islet phenotype of MyD88−/−/Trif−/− mice would mimic the Trif−/− phenotype as would the underlying mechanism. Further studies are required to validate this hypothesis, and also to determine whether there is crosstalk between MyD88 and TRIF in regulating glucose tolerance and glucose stimulated insulin secretion. MyD88 signalling was observed to negatively regulate TLR3/TRIF activation in human corneal epithelial cells through the inhibition of JNK; however, this finding was not observed when tested in macrophages [280]. While this observation highlights a mechanism for crosstalk between TRIF and MyD88, it also points to the possibility of altered MyD88 function in epithelial cells versus macrophages and potentially beta cells.

Interestingly, it seems that the role of TRIF in glucose homeostasis and beta-cell function may be more important than that of MyD88. This possibility is seemingly counterintuitive as MyD88 plays a bigger role in inflammation, acting as the sole signalling molecule for almost all TLRs and the IL-1R. This points to the possibility that TRIF may play a role in glucose homeostasis, independent of the TLR pathway. Inherent defects in
beta-cell secretion exist and further experiments are required to determine which intracellular pathways within the beta cell are affected by TRIF deficiency

### 4.3.4 Islet cell types involved in inflammation

The cell type responsible for the phenotype seen in \( \text{MyD88}^{/-} \) and \( \text{Trif}^{/-} \) is also important to discuss. As this study uses global gene knockout or loss of function mutation mice, one cannot assume that effects on glucose tolerance or even islet glucose-stimulated insulin secretion are strictly due to differences in beta-cell function. The islet is comprised of multiple cell types including beta cells, alpha cells, delta cells, endothelial cells, macrophages and dendritic cells. Sorted beta cells secrete significantly less chemokine (MCP-1, CXCL10, CCL5) under basal and stimulated conditions compared to non-beta-cells [185]. Also, depleting dispersed islets of vascular endothelial and dendritic cells drastically reduces MCP-1 and CXCL10 expression [185]. These results provide evidence that multiple cell types coordinate islet inflammatory responses, and beta cells may only play a minor role. Lack of TLR signalling in other cell types may therefore induce functional differences within the islet or perhaps other sites of glucose homeostasis, and thus lead to the observed phenotypes.

Endothelial cells are important for beta-cell proliferation and function [281]. Glucose-stimulated insulin secretion and islet insulin content were found to be enhanced after exposure to endothelium conditioned medium, and this effect was proposed to be mediated through laminins [282]. Endothelial cells have also been proposed to mediate the harmful effects of free fatty acids and glucose on islets via the production of ROS. Circulating glucose and free fatty acids activate islet endothelium, inducing ROS and the production of inflammatory cytokines, which in turn activates macrophages, causing islet infiltration and further production of harmful cytokines [283]. Lack of TLR signalling during hyperlipidemia and hyperglycemia may protect from ROS production; however, under normal conditions, lack of TLR signalling may influence endothelial cell function and contribute to observed islet dysfunction.

Resident macrophages are essential in mediating intra-islet inflammatory responses in both type 1 and type 2 diabetes [18, 130]. The phenotype of resident macrophages changes during disease, and in obesity, adipose tissue macrophages switch from the alternative M2 phenotype to the classical inflammatory M1 phenotype [284]. In lean mice adipose tissue
macrophages secrete IL-10, protecting against insulin resistance, and this same protection may occur with resident islet macrophages [284]. TLR ligation may be important in polarizing macrophages towards the M2 phenotype, and disruption of this polarization could result in islet dysfunction. Recent evidence suggests that this may not be the case, however, as PPAR-γ is essential for the maturation of alternative activated macrophages and activation of PPAR-γ results in decreased TLR expression and activity in monocytes [285, 286]. Generating bone-marrow chimeras using TLR (e.g. TLR2 or TLR4) or TLR signalling molecule (e.g. TRIF or MyD88) knockout animals would be a useful model to determine the role of TLR signalling in bone-marrow derived tissues in glucose homeostasis. Furthermore, tissue specific deletion of TLR signalling pathways could be achieved using the Cre-loxP system using the CD11c promoter for bone-marrow derived macrophages and dendritic cells [287, 288].

4.3.5 Impact of TLR signalling and gut flora on metabolic homeostasis

The gut is an important site of metabolic homeostasis as well as immunological tolerance. The intestinal barrier is responsible for maintaining tolerance toward beneficial gut microbiota, while constantly surveying for potentially pathogenic bacteria. The intestinal epithelium consists of TLR-expressing epithelial and dendritic cells, and below the epithelial layer, plasma cells, macrophages and myofibroblasts also express TLRs [289]. The responsiveness of TLR2 and TLR4 in intestinal epithelial cells is muted, leading to LPS tolerance and cross-tolerance to other pathogen-associated molecular patterns [289]. Decreased activity of these TLRs is related to decreased surface TLR expression, as well as increased expression of TLR-inhibitory molecule, Tollip. TLR5 is essential for maintaining gut homeostasis, as lack of TLR5 induces the development of spontaneous colitis [290]. Other pattern recognition receptors also play a role in the gut homeostasis; NOD1 is responsible for responding to gram-negative bacteria such as Shigella flexneri while NOD2 can respond to peptidoglycans present in both gram-positive and negative bacteria [289]. As TLR signalling is essential for maintaining gut barrier integrity, it is important to consider the impact of the gut microbiota in the present study as a potential primary or secondary mechanism underlying the observed phenotype in Trif−/− and MyD88−/− mice.
Increased intestinal permeability is associated with obesity and diabetes in mice, and diet-induced changes in gut microbiota can contribute to the onset of obesity and insulin resistance [291-293]. Metabolic endotoxemia is a condition of elevated circulating LPS levels, and it is associated with high-fat diet-induced hyperglycemia and insulin resistance [291]. Inducing metabolic endotoxemia in mice by administering low-doses of LPS can induce hyperglycemia, hyperlipidemia and hepatic insulin resistance, as well as increased adipose macrophage infiltration, pointing to the role of LPS in potentially mediating or initiating diet-induced metabolic syndrome [291]. Lack of TLR5 in mice induces changes in the gut flora, leading to hyperinsulinemia, hyperglycemia and glucose intolerance [221]. This altered gut flora was capable of inducing metabolic syndrome in germ-free, wild-type mice, pointing to the role of gut flora composition in controlling inflammation and potentially metabolic syndrome. Therefore, changes in circulating LPS levels and gut flora, induced by lack of TRIF or MyD88 signalling may be affecting glucose homeostasis in Trif−/− and MyD88−/− mice. As TLRs are important for maintaining gut homeostasis [294], lack of TRIF or MyD88 signalling may induce a shift in gut tolerance, leading to chronic inflammation, disrupting both gut tolerance and glucose homeostasis. The use of antibiotics to protect MyD88−/− mice from opportunistic infection may have muted or dampened the manifestation of a metabolic syndrome phenotype. Antibiotic treatment may have controlled the overgrowth of commensal bacteria in MyD88−/− mice, preventing an imbalance in gut tolerance and dysregulated inflammation.

The gut serves as an important interface between metabolic homeostasis and innate immunity. It may also serve as a common crossover point between type 1 and type 2 diabetes, as gut microbiota has been shown to be important for the development of type 1 diabetes in MyD88-deficient NOD mice [193]. The gut microbiota is essential for growth and development and increased intestinal permeability is associated with a susceptibility to autoimmune disease in humans [195, 196]. A hypothetical model for the contribution of gut “leakiness” to beta-cell autoimmunity is that a leaky gut leads to increased permeability to dietary antigens and cytokine release, leading to the induction of autoreactive T cells [196]. In type 2 diabetes, a similar event could be taking place such that over time intestinal permeability deteriorates, leading to systemic chronic inflammation, and subsequent dysfunction in metabolic homeostasis. Therefore, in this study, it is important to consider the
contribution of lack of TLR signalling in the intestinal epithelium to the observed phenotype in $\text{Trif}^{-/-}$ and $\text{MyD88}^{-/-}$ animals.

4.3.6 Future directions for the role of TLR signalling in normal beta-cell function

This study provides evidence that TLR signalling via TRIF and MyD88 may play a role in normal beta-cell function and glucose homeostasis under non-pathological conditions. One limitation of this study is the use of global loss of function or gene knockout mice, and the impact of TRIF or MyD88 deficiency on other sites of glucose homeostasis such as the gut, hypothalamus, muscle, liver and adipose tissue remains to be established. Metabolic homeostasis involves specific, regulated coordination of signals between these organs, and due to the wide expression pattern of TLRs and their signalling molecules, it is essential to perform follow-up studies with cell-specific knockout mouse models.

With the advent of the $\text{MyD88}$-loxP mouse [288], tissue-specific gene expression of MyD88 can be controlled, allowing for future studies to examine the role of MyD88 in the beta cell, and other islet cell types, in both development and adulthood. Using the inducible PDX-1-Cre model [295, 296], $\text{MyD88}$ expression can be controlled in adulthood, preventing unwanted developmental side effects due to MyD88-deficiency during embryogenesis. Establishing the role of MyD88 in beta-cell function in response to a high-fat diet is essential for understanding how TLR and IL-1R pathways impact glucose homeostasis in response to metabolic changes. With the cell-specific knockout model, evidence can be generated as to which cell type is the primary contributor of TLR and IL-1R induced islet inflammation.

Currently, a $\text{Trif}$-loxP mouse has not been created; however, vector microinjection experiments in mice are underway at the Wellcome Trust Sanger Institute (Hinxton UK), a genome research center with a high-throughput gene targeting facility. Once created, a beta-cell specific TRIF knockout mouse could be useful for establishing the mechanism behind the observed phenotype in $\text{Trif}^{-/-}$ mice; using littermate controls, high-fat diet studies could be repeated, as well as further studies looking into beta-cell glucose-stimulated insulin secretion. Also, further analysis is required to investigate the effect of NAIP2 deficiency on beta-cell function. NAIP2 may be important for sensing inflammation within the beta cell in response to oxidative or ER stress, and further studies are required to examine the function of NAIP2 in beta cells, as well as the expression pattern of NAIP2 in other areas of glucose.
homeostasis. Aside from the beta cell, site-specific manipulations of MyD88 and TRIF expression in the gut, liver, muscle, adipose tissue and hypothalamus may help to further elucidate the role of TLRs in metabolism.
CHAPTER 5: CONCLUSIONS

Inflammation is an important, underlying feature of type 1 and type 2 diabetes. Controlling the generation and amplification of innate and adaptive immune responses in islet transplantation and type 2 diabetes may greatly improve disease outcomes. Upon endogenous or exogenous stimulation, innate immune responses drive the polarization of adaptive immunity, and TLRs play an essential role in managing this process. Endogenous danger signals are increased during infection and injury, and preventing the amplification of immunity in response to danger signals can greatly improve pathogenic inflammation.

Inflammatory cells and cytokines can be beneficial in normal physiology, as inflammation is required for tissue repair and regeneration. Similarly, lower levels of inflammation can enhance glucose-stimulated insulin secretion and alpha-cell proliferation within the islet [145, 297]. However, during disease pathogenesis, hyperglycemia and injury initiates a proinflammatory environment, increasing the expression of proinflammatory cytokines and the expression of TLRs on monocytes [298, 299]. TLR activation in response to increased danger signals may exacerbate allograft rejection and diabetes-induced islet dysfunction. With such a vast array of endogenous ligands, targeting TLRs for therapy could prevent the manifestation of the diabetes-associated inflammatory state, and therefore improve disease management. However, TLR function during normal conditions may also be important for regulating glucose homeostasis, and play a role in metabolic maintenance.

An important finding of this project is that islet allograft rejection occurs independently of TLR4 signalling. While TLRs are important for contributing to T-cell responses, these findings suggest that in allograft rejection, TLR activation may play only a supporting role in preventing tolerance and promoting graft dysfunction. Targeting TLR pathways for inhibition in allograft rejection may prevent primary graft dysfunction and alleviate the need for intense, long-term immunosuppression. As TLR activity in the host seems to play an important role in allograft tolerance, developing pre-transplantation therapies against TLR-induced inflammation may also extend graft longevity. As allograft rejection involves a coordinated response among multiple systems, it seems appropriate that blockade of one family of receptors would not completely abrogate allograft rejection.
However, the importance of TLR signalling should not be underestimated, as innate immunity is important in establishing tolerance, and controlling regulatory T-cell action.

In the second part of this study, our findings indicate that although TLR signalling in chronic inflammation is detrimental to the beta cell, TLR signalling pathways may play an essential role in glucose homeostasis and normal beta-cell function (Figure 31). These findings are somewhat surprising as it implies that TLR signalling is important for maintaining glucose homeostasis, outside of pathological inflammation. While TLR signalling is important during infection and injury, and can promote islet inflammation and dysfunction, it seems that TLR function is also important to the beta cell under normal conditions, and may contribute to or mediate the benefits of cytokine action for the beta-cell. Current evidence suggests that low levels of cytokines (e.g. IL-1β and IL-6) contribute to glucose homeostasis, and TLR signalling via TRIF and MyD88 may be a mechanism for the stimulation of these cytokines under normal conditions.

Determining the mechanism of activation and regulation of TLR activity in non-pathological states is essential for understanding how innate immunity contributes to glucose homeostasis. It is well established that hyperglycemia is associated with both acute and chronic infection, and interestingly, glycemic control can reduce morbidity and mortality among critically ill patients [300, 301]. As well as increasing TLR expression, hyperglycemia also increases the formation of advanced glycation end-products (AGEs). AGEs are non-enzymatically glycated or oxidized proteins, lipids and nucleic acids that are formed under conditions of oxidative stress or hyperglycemia [164]. AGEs can propagate inflammation through TLRs and RAGE. Metabolic endotoxemia is a state of elevated circulating LPS levels that occurs after high-fat feeding, and maintaining high levels of circulating LPS can induce hyperglycemia, hyperinsulinemia and insulin resistance in mice [291]. Circulating levels of LPS are elevated in patients with type 2 diabetes, and LPS levels correlate strongly with blood glucose [302]. Therefore, while high levels of LPS, glucose, AGEs or lipids may promote inflammation in obesity and type 2 diabetes, these molecules may also be involved in controlling metabolism under normal conditions. At a certain threshold, the concentration of these mediators may induce toxicity; however, at low levels, TLR activation may promote regulation and glucose homeostasis.
Figure 31: Innate immunity may be essential for normal beta-cell function and glucose homeostasis. Under non-pathological conditions, innate immune activity may contribute to regulatory pathways for beta-cell function and glucose homeostasis. Adapted from [225].
An expansion of the Danger Model proposed by Matzinger [162] suggests that tissues contribute to immune responses, providing a polarizing environment for the type of immune response required. However, in addition to pathogenic immune responses, the immune system acts as a physiological means of communication. Interactions between resident (e.g. macrophages and dendritic cells) and circulating immune cells may be responsible for transmitting homeostatic signals. These signals may be essential for metabolic maintenance and communication between sites of glucose homeostasis (e.g. pancreas, liver, adipose tissue). Innate immune signalling induced by glucose, LPS, lipids or AGEs may modulate hypothalamic leptin action, hepatic glucose production, lipolysis and/or insulin secretion. For example, non-pathological levels of LPS and glucose stimulate low levels of IL-1β, which in turn enhance glucose-stimulated insulin secretion [145]. In response to high-fat diet, increased LPS and glucose levels lead to an increase in IL-1β, contributing to further hyperglycemia. Levels of metabolic mediators (e.g. glucose, LPS, lipids or AGEs) may dictate the nature of innate immune interactions within the glucose homeostatic cascade. As the concentration of metabolic mediators over time reaches or spikes above threshold levels, these signals may switch from regulatory to proinflammatory.

This hypothesis may explain the results reported here. Lack of TLR signalling via TRIF resulted in hyperglycemia and glucose intolerance. This phenotype could result from impaired communication between TRIF-deficient immune cells and other systems leading to dysregulated hepatic glucose production, lipolysis, and glucose uptake. Also, mice lacking TRIF did not adapt as well as control mice to a high-fat diet, suggesting that TRIF signalling may be essential for global metabolic adaptation. One could hypothesize that the threshold for the phenotypic switch between regulation and proinflammation varies between individuals, and may explain why some obese patients do not develop type 2 diabetes.

These metabolic mediators of innate immunity may also promote autoimmunity. Inappropriate activation of the innate immune system or high sensitivity to these mediators at otherwise non-pathological levels may be a trigger for the development of beta-cell autoimmunity. This inappropriate activation could be the result of a genetic predisposition to a proinflammatory state, resulting in chronic inflammation and autoimmune destruction.

While the innate immune response may be detrimental to islet homeostasis during disease and infection, it may also play a role in islet maintenance and metabolic adaptation
under non-pathological conditions. Although not required for islet allograft rejection, TLR signalling is important for immune regulation and early graft function in islet transplantation. Also, TLR signalling via TRIF and MyD88 is important for maintaining normal blood glucose levels, glucose tolerance, and glucose stimulated insulin secretion. These studies provide new insight into a role for TLR signalling in islet transplantation and diabetes.
REFERENCES


APPENDICES

Appendix A: Laboratory Certificates

UBC Animal Care Certificate
UBC Biohazard Approval Certificate
ANIMAL CARE CERTIFICATE

Application Number: A06-1452

Investigator or Course Director: C. Bruce Verchere

Department: Pathology & Laboratory Medicine

Animals:

- Mice Toll-like receptor 4 (TLR4) knockout 34
- Mice CD11c-DTR/EGFP NOD.scid 36
- Mice TetO-CMV-XIAP transgenic 8
- Mice RIP-rTA / TetO-CMV-XIAP bigenic 8
- Mice Toll-like receptor 3 (TLR3) knockout 34
- Mice Balb/c 72
- Mice RIP-rTA transgenic 8
- Mice TICAM-1 Knockout 60
- Mice MyD88 Knockout 34
- Mice Bl/6 344
- Mice CD11c-DTR/EGFP 36
- Mice Apolipoprotein E knockout 30
- Mice NOD 49
- Mice NOD.scid 40

Start Date: September 1, 2006  Approval Date: March 26, 2009

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Prolonged protection of islet allografts by inhibition of beta cell apoptosis
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
Biohazard Approval Certificate

PROTOCOL NUMBER: B09-0088

INVESTIGATOR OR COURSE DIRECTOR: C. Bruce Verchere

DEPARTMENT: Pathology & Laboratory Medicine

PROJECT OR COURSE TITLE: Islet amyloid and islet transplant failure

APPROVAL DATE: October 15, 2009 START DATE: July 1, 2008

APPROVED CONTAINMENT LEVEL: 2

FUNDING TITLE: Amyloid-Based Therapies for Type 2 Diabetes
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Centre for Human Islet Transplant and Beta Cell Regeneration (CHITBR)
FUNDING AGENCY: Michael Smith Foundation for Health Research

FUNDING TITLE: Islet Amyloid And Islet Transplant Failure
FUNDING AGENCY: Canadian Diabetes Association

UNFUNDED TITLE: Islet amyloid and islet transplant failure

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
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Appendix B: List of Publications
