PREVENTION OF TYPE 1 DIABETES BY CARBAMAZEPINE IN NON-OBESE DIABETIC MICE

by

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Abstract

Pancreatic β cells are selectively destroyed by the host immune system in type 1 diabetes, which results in the inability to regulate glucose homeostasis due to loss of insulin production capacity. Drugs that preserve β cell mass and function therefore have the potential to prevent or slow the progression of this disease. It was recently reported by our group that the use-dependent sodium channel blocker, carbamazepine, protects pancreatic β cells from inflammatory cytokines in vitro. Subsequent experiments found carbamazepine increased insulin gene expression, which corroborated with an increase in insulin content in islets from mice lacking the Nav1.7 voltage gated sodium channel, which was shown to be a target of carbamazepine in β cells. While these in vitro results were promising, it was unclear whether carbamazepine would protect β cells in vivo against a complete immune system. Therefore, we tested the effects of oral treatment in female non-obese diabetic (NOD) mice, achieving serum carbamazepine levels of 14.98 ± 3.19 µM. Remarkably, diabetes incidence over 25 weeks was ~50% lower in carbamazepine treated animals. Partial protection from diabetes in carbamazepine-fed NOD mice was also associated with improved glucose tolerance at 6 weeks of age, prior to the onset of diabetes in our colony. Insulitis was improved in carbamazepine treated NOD mice at 6 weeks of age, but we did not observe differences in CD4+ and CD8+ T cell composition in the pancreatic lymph node, as well as circulating markers of inflammation. Taken together, our results demonstrate that carbamazepine reduces the development of type 1 diabetes in NOD mice.
Lay Summary

Type 1 diabetes is a condition where the body's own immune system attacks the cells in the pancreas called β cells that make the hormone insulin. This results in high blood sugar and leads to many secondary diseases. The Johnson lab used automated ways of searching for drugs that protect β cells from death in type 1 diabetes-like conditions and found that a drug called carbamazepine, FDA approved for epilepsy, is effective. In the present study, we confirmed that carbamazepine significantly reduces type 1 diabetes in mice. Follow up experiments found improved responses to glucose challenges, and a decrease in immune cells attacking the β cells in young mice. Taken together, these results suggest that carbamazepine could potentially be useful in the treatment of type 1 diabetes.
Preface

I designed, performed, and analyzed all experiments within this thesis under the supervision of Dr. James D. Johnson. Iryna Shanina and Dr. Marc S. Horwitz provided the non-obese diabetic (NOD) mice and expertise related to working with the NOD mouse model. Angel Yung Ning Chu assisted with immunohistochemical staining of pancreatic sections, and Betty Hu and Derek Dionne assisted with in vivo metabolic experiments performed on a chase cohort of NOD mice.

The following data presented in Chapter 3 is currently in the process of submission to a scientific journal.

All in vivo experiments were approved by the UBC Animal Care Committee protocols A11-0390 and A14-0197 listed under principal investigator Dr. James D. Johnson.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APECED</td>
<td>Autoimmune polyendocrine syndrome type 1 (also known as APS-1)</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>APS-1</td>
<td>Autoimmune polyendocrine syndrome type 1 (also known as APECED)</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CGM</td>
<td>Continuous glucose monitoring</td>
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<tr>
<td>CVB</td>
<td>Coxsackie virus</td>
</tr>
<tr>
<td>DAISY</td>
<td>Diabetes and Autoimmunity Study in the Young</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamidine-2’-phenylindole dihydrochloride</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<tr>
<td>DIPP</td>
<td>Type 1 Diabetes Prediction and Prevention Project</td>
</tr>
<tr>
<td>DiViD</td>
<td>Diabetes Virus Detection Study</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
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<tr>
<td>GWAS</td>
<td>Genome wide association studies</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon γ</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL2RA</td>
<td>Interleukin 2-receptor alpha chain</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T</td>
</tr>
<tr>
<td>LADA</td>
<td>Latent autoimmune diabetes in adults</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
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<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
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<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<tr>
<td>RIP</td>
<td>Rat insulin promoter</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage gated sodium channels</td>
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Acknowledgements

The last two years of graduate school concludes my seven-year post-secondary journey of education at UBC. To my two close family members that left a little too soon, thank you for motivating me to pursue a medical career. What started as inklings of curiosity in biomedical science and a desire to help others has flourished into a passion and respect for the great work that scientists do to advance their respective fields every single day.

A very special thanks to my mentor, Dr. James D. Johnson, for providing an unbridled enthusiasm and curiosity for science that got me through many a failed experiment. It is absolutely astounding how much time you’ve dedicated to helping me through everything, and I am truly grateful for all the support you’ve given me over the years.

Needless to say, much of this work could not have been done without the amazing support of all my fellow lab mates. In the past four years, I’ve had the pleasure of watching six graduate students and three postdoctoral fellows move on to bigger and better futures, and I am happy to say that I consider each and every single one a treasured friend and colleague. This applies equally to those still in the lab, and I can honestly say that the hardest part of leaving the lab will be having to give up working with such a wonderful and amicable group of friends and colleagues. My heartfelt thanks to you all.

Lastly, a big thank you to my friends, my lovely partner, and my family. Although this seems to be somewhat of an almost overused trope, I really could not have done it without you all. As I embark on my professional career in a place a little more distant than where I am now, please know that my successes are a reflection of all the hard work and support that you’ve given me. I stand proudly on your efforts, knowing that whatever I accomplish in the future is a direct result of all the hard work and support you’ve given me.
To friends and family, and all those who suffer from disease on a daily basis.
Chapter 1: Introduction

1.1. Overview of Type 1 Diabetes Pathology

1.1.1. Background of Type 1 Diabetes

According to the 2016 World Health Organization’s “Global Report on Diabetes”, approximately 422 million adults in 2014 were classified as diabetic, and rates of diagnoses have steadily increased over recent years (1). Costs associated with diabetes care worldwide are estimated at $827 billion USD yearly (2). The two most prevalent forms of diabetes are classified as type 1 and type 2, where they make up approximately 10% and 90% of all known cases respectively (3). Other types of diabetes, including gestational diabetes and monogenic diabetes, also known as maturity onset diabetes of the young (MODY), occur at a much lower rate, making up approximately 1% of all known cases (3; 4).

Type 1 diabetes is an autoimmune disorder in which pancreatic β cells are selectively destroyed by the host immune system, leading to the loss of insulin production capacity and subsequent blood sugar dysregulation (5). Type 1 diabetes was previously referred to as ‘juvenile-onset diabetes’ based on the prevalence of diagnosis in younger patients (mean age of diagnosis at 14 years old), although this term has fallen out of favour due to the fact that patients can also develop autoimmune diabetes later in life (6). This disorder is commonly referred to as latent autoimmune diabetes in adults (LADA) and shares similar pathological characteristics as classical type 1 diabetes, but can be diagnosed well past the age of 40 (6). Moreover, non-autoimmune type 2 diabetes is increasingly being diagnosed in children, which makes the designation of ‘juvenile-onset diabetes’ even more ambiguous (7; 8).

Primary complications of type 1 diabetes, when left untreated, are attributed to chronic hyperglycemia, where the ability of a patient to control day-to-day blood sugar is correlated to the
incidence of diabetes-associated pathologies (9-11). Complications typically involve damage to the microvasculature, and includes the development of retinopathy, nephropathy, neuropathy, and cardiovascular disease (12). Hyperglycemia can also delay wound healing and increase the risk of infection (12). Although the prevalence of each complication varies with the relative population and severity of disease, they contribute as a whole to increase mortality in the diabetic patient populace (12). ‘Intensive approaches’ to control glycemia as defined by “three or more daily injections of insulin or treatment with an external insulin pump” as compared to ‘conventional therapy’ with “no glucose goals beyond those needed to prevent symptoms… and consisted of one or two daily injections of insulin” studied in the Diabetes Control and Complications Trial (DCCT) encouragingly found a reduction in cardiovascular events and micro-albuminuria as compared to conventional therapeutic regimens (13), but this was also associated with a two to six-fold increase in acute hypoglycemic episodes (14). Severe hypoglycemic episodes are undesirable, as they can either cause immediate death if left untreated, or have also been known to be associated with an increase in the development of cardiovascular and non-cardiovascular comorbidities (15). This speaks to a need for optimization of current therapeutic regimens and the discovery of alternative therapies.

1.1.2. Genetic Risk Factors for Type 1 Diabetes

As of today, the exact etiology of type 1 diabetes remains unclear, although the broad outlines have been sketched out over decades of research. Both genetic and environmental factors seem to contribute to the development of type 1 diabetes, but definitive casual factors have not yet been identified. According to the worldwide Diabetes Mondiale study, higher instances of type 1 diabetes are associated with certain racial and ethnic backgrounds (e.g. high incidence of diabetes
amongst Finnish people), which allows scientists to study at risk populations to identify genetic variants that contribute to the development of type 1 diabetes (16). Although the study of certain racial backgrounds to identify risk loci for type 1 diabetes is promising, genetically identical (monozygotic) twins did not necessarily develop diabetes even if their sibling did (17-19). The proportion of Finnish twins that co-develop type 1 diabetes was at the maximum, approximately 34% by 12 years of age (17-19). Furthermore, it was concluded that past the age of 12, only an additional 2% continue to develop type 1 diabetes, which suggests causative factors outside of genetics alone (17).

Early genetic linkage analysis in humans reported single nucleotide polymorphism (SNP) variants associated closely with genes and regulatory elements linked to type 1 diabetes (20). In total, 18 different genetic loci were initially identified that exhibited association to type 1 diabetes (20). The most prominent loci associated with type 1 diabetes was identified as the human leukocyte antigen (HLA) genes on chromosome 6p21 (IDDM1) (21). Subsequent analyses found that these SNPs most likely corresponded to the genes HLA-DR and HLA-DQ, where both susceptible and protective alleles were identified (22). HLAs are a part of the class II type major histocompatibility complex (MHC), which are responsible for presenting antigens from professional antigen presenting cells (APCs) to T cells to mediate their activation. Later studies also found SNP variants associated with MHC class I genes such as HLA-A and HLA-B (23). Class I MHCs are responsible for presenting antigen from within the cell, especially during viral infection, and are expressed by most of the tissues in the body. Together, these factors constitute a major proposed driver of type 1 diabetes pathogenesis.

Genes outside of the HLA loci have also been linked to type 1 diabetes, although the associations between these genes and disease are weaker. These genes were identified primarily
through candidate gene-association studies, where genes of relevance to antigen presentation and immune function were specifically queried to link them to identified risk loci (20). Some examples of candidate genes that were identified to associate with risk loci include: *INS, CTLA4, PTPN22*, and *IL2RA* (20).

There is strong evidence that insulin itself, and therefore the pancreatic islets, play a non-passive role in common forms of type 1 diabetes. For example, insulin is believed to be the primary autoantigen in type 1 diabetes (24; 25). Genetic variation around the *INS* gene locus (IDDM2) is primarily associated with the promoter rather than the gene itself, in a region with a variable number of tandem repeats (VNTR). The shorter the repeats, the greater the risk for development of type 1 diabetes (26). Experiments suggest that the VNTRs are responsible for modulating insulin expression level, and shorter repeats reduce levels in the thymus analogous to mutations in *AIRE* (see below), while paradoxically increasing the production of insulin in the pancreatic islets (27; 28). Additional studies are necessary to determine whether this increase in insulin production in islets contributes as a causal factor in the pathogenesis of type 1 diabetes.

CTLA4, also known as CD152 (IDDM12), is best known as an immune checkpoint protein similar in structure to the co-stimulatory protein CD28 (24). CTLA4 acts to inhibit T cells as opposed to activating them however, and is commonly expressed on regulatory (Tregs) and already activated T cells for negative feedback (24). An A49G mutation in CTLA4 results in improper processing within the ER, resulting in decreased surface expression and is correlated to the development of type 1 diabetes (29). Two additional loci were linked to the genes *PTPN22* and *IL2RA*, corresponding to the lymphoid protein tyrosine phosphatase (LYP) and interleukin 2 receptor. Collectively, these proteins play a role in T cell signaling and Treg development, two processes important in the pathogenesis of type 1 diabetes (24).
With the advent of genome wide association studies (GWAS), many of the identified genes replicated the results originally identified in candidate linkage analysis (20). GWAS also greatly expanded our knowledge of risk loci, which provided scientists with additional mechanisms of study in type 1 diabetes (20). One notable example is IFIHI or MDA5, an interferon-induced helicase used to identify picornavirus infection, which is believed to be a major environmental risk factor for type 1 diabetes (30). Interestingly, although many SNPs associated with MDA5 were associated with development of diabetes (31), rare variants were also identified in subsequent studies that were found to be protective (32). Collectively, these genes are associated with foreign antigen recognition, identifiable type 1 diabetes autoantigens, and negative regulators of immune function and provide plausible leads in the investigation of causal and disease-accelerating factors in type 1 diabetes (20; 24).

1.1.3. Single Genes Affecting Immune Cell Maturation and Autoregulation

Autoimmune diabetes can also be caused by single mutations in genes, but these conditions are relatively rare. Most cases of type 1 diabetes are believed to have both an environmental and a genetic component which act in tandem to bring about disease. Examples of monogenic autoimmune diabetes include autoimmune polyendocrine syndrome type 1 (APS-1 or APECED) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (33; 34). APECED refers to mutations in AIRE, a transcription factor important in allowing medullary thymic epithelial cells (mTECs) to express autoantigens in T cell development (34). Approximately 20% of children that harbor mutations in AIRE will go on to development autoimmune diabetes (34). IPEX syndrome refers to mutations in the FOXP3 gene, which is also a transcription factor important in the function of regulatory T cells (33). These children experience
high incidences of type 1 diabetes of approximately 80%, and most do not make it into adulthood due to a wide variety of disorders associated with systemic dysfunction of the immune system (35). Although these mutations are rare in the population of patients with type 1 diabetes, they remain a promising area of study for scientists seeking to understand autoimmunity.

1.1.4. **Immune Cell Maturation Defects in Type 1 Diabetes**

Defects in maturation of immune cells are the principal cause of APECED (34), and studies on aberrant negative and positive selection of T cells leading to autoimmunity have been well documented in literature (34). Central and peripheral tolerance are terms that refer to the mechanisms by which developing T and B cells are selected for reactivity against foreign antigen and discarded for reactivity against auto-antigens. Because all T and B cells are genetically identical, reactivity of the cells to different antigens depends on the random combinatorial arrangement of several gene segments to create unique T (TCR) and B (BCR) cell receptors. T and B cells are aptly named according to the lymphatic organ in which they mature, in the thymus and bone marrow respectively. Because type 1 diabetes is primarily attributed to being a ‘Th1-skewed’ or pro-inflammatory disease, most of the current scientific literature is focused on the cytotoxic T cell as opposed to the antibody-producing B cell. Studies have shown that B cells are not required for the development of type 1 diabetes, but the presence of B cells and the ability to secrete antibodies exacerbates the incidence of the disease (36).

1.1.5. **Thymic Selection in Type 1 Diabetes**

The presence of autoreactive T cells that destroy pancreatic β cells are attributed to defects in their selection early in development (37). As the naïve T cell enters the thymus, it undergoes a
series of selection originating in the thymic cortex (38). Binding affinity of the naïve cell to a surface MHC molecule expressed in high quantities by the thymic epithelial cells is assessed in a process known as positive selection (38). If the T cell is able to bind successfully to an MHC molecule, it is spared from programmed cell death and transitions from being a double positive CD4⁺CD8⁺ cell to either a CD4⁺CD8⁺ or CD4⁺CD8⁻ cell based on its ability to bind MHC class I or II molecules (38). This process determines its developmental fate as a helper T or a cytotoxic T cell. T cells then undergo negative selection, in which mTECs express autoantigens (such as insulin) driven by specialized transcription factors like AIRE, where the cells that bind with strong affinity are induced to undergo programmed cell death to mitigate autoimmunity (38). Experiments involving the thymic deletion of the Ins2 gene in the C57B6/L background developed severe hyperglycemia by three weeks of age in both male and female mice (37). Insulitis, defined as the infiltration of immune cells into the islet, comprised of both CD4⁺ and CD8⁺ T cells was readily detectable in these animals, and adoptive transfer of either cell type into immunodeficient Rag1⁻/⁻ mice caused diabetes (37). However, it should be noted that, in another series of studies, complete knockout of the Ins2 gene did not result in type 1 diabetes (39; 40). Use of a double transgenic RIP-mOVA/OT-I TCR mouse model where ovalbumin expression is driven under the rat insulin promoter (RIP) with concurrent production of a CD8⁺ T cell clone specific for ovalbumin resulted in spontaneous diabetes only in the absence of Aire (41). Notably, the global knockout of Aire has been shown to cause the development of a systemic, non-specific autoimmune disorder with detectable serum autoantibodies (27). The effect of Aire knockout also seems to be exacerbated in the non-obese diabetic mouse model as opposed to the C57B6/L background, where additional organs are targeted and infiltration of mononuclear cells is more aggressive (42). Interestingly, knockout of Aire in the NOD mouse model resulted in protection from diabetes, where acinar cells
rather than β cells were destroyed, and autoantibodies were detected for pancreas-specific protein disulfide isomerase (PDIp), a protein expressed primarily by acinar cells (43). In a similar vein, studies with Deaf1, similar in structure to Aire, in the pancreatic lymph node found its influence in the expression of peripheral tissue antigens (44). Similarly, expression of insulin in peripheral lymphatic tissues are important for the maintenance of immune tolerance, as the knockout of Ins2 results in increased proportion of T cell activation and induction of diabetes in a subset of male mice (45). Collectively, this evidence suggests that dysfunctional ‘education’ of immune cells early in development play a role in autoimmune disorders like type 1 diabetes.

1.1.6. Peripheral Tolerance by Regulatory T Cells in Type 1 Diabetes

Studies with the NOD mouse model provided evidence that there was an additional CD4+ T cell sub-type that functioned as an immunoregulator, due to the discrepancy between detectable insulitis within 3 weeks of age versus the relatively drawn-out induction of β cell destruction over the timespan of months (46). These T cells are referred to as regulatory T cells (Tregs), and are vitally important in dampening inflammatory responses (46). Murine Tregs are identified by the expression of CD4, CD25 and FOXP3, where the expression of CD62L at high levels also indicates its relative level of potency (47). These cells can also be developed peripherally, and experiments on murine tissues in vitro have confirmed that stimulation of naïve T cells with an antigen corresponding to the TCR in the presence of transforming growth factor β (TGF-β) will also push them towards Treg lineage (48). Notably, experiments involving adoptive transfer or the expansion/development of antigen-specific Tregs ameliorated the development of type 1 diabetes in several different diabetic mouse models (49-52). Evidence in human suggests that Tregs isolated from patients with type 1 diabetes are defective and unable to suppress pro-inflammatory
environments (53). Expansion of Tregs in humans as an immunotherapy has been a promising area of study to dampen autoimmune responses and preserve β cell mass (54).

1.1.7. Environmental Triggers of Type 1 Diabetes

Many different environmental triggers of type 1 diabetes have been proposed, but none have been shown to reliably trigger and fully emulate the pathogenesis of human type 1 diabetes in a physiological manner. It is also unknown how these proposed factors act in tandem with genetic risk factors to cause diabetes. Proposed environmental contributors to the development of type 1 diabetes include: immunogenic and/or cytotoxic viral infection, viral antigen mimicry, bystander damage, and pro-diabetic and anti-diabetic contributions of intestinal microbiota.

Viruses and early viral infection have been postulated to be causal or contribute towards the development of type 1 diabetes. Proposed mechanisms for pathogenesis include: direct pancreatic β cell cytolysis, promoting pro-inflammatory environments, and inducing ‘antigen mimicry’, whereby proteins from the virus cause cross-reactivity to antigens found on the pancreatic β cell and subsequently activate the immune system to mistakenly attack them (55). Of the many types of viruses that have been proposed to contribute to type 1 diabetes pathogenesis (e.g. rotavirus, retrovirus, picornavirus), most scientific studies focus on a subset of enteroviruses that belong to the picornavirus family, Coxsackie virus. Enteroviruses are notable for their tropism towards the cells of the endocrine pancreas (56; 57), and initial studies found a strain of Coxsackie B4 (CVB4) virus in the pancreas of a recently diagnosed type 1 diabetes patient, where subsequent infection of SJL mice, known to be defective in suppressor T cell development, produced notable hyperglycemia as compared to C57B6/L mice (58). Similarly, experiments examining the transplantation of human islets in β cell ablated mice found that CVB4 challenge resulted in
persistent hyperglycemia and decreased insulin production (59), similar to enterovirus-infected human islets (57). Unfortunately, correlation studies investigating the diagnosis of diabetes and presence of enterovirus RNA in blood and stool, such as the Norwegian Environmental Triggers of Type 1 Diabetes (MIDIA) and the Diabetes and Autoimmunity Study in the Young (DAISY), were unsuccessful in finding a strong link between CVB4 and type 1 diabetes development (60-62). Interestingly, the Type 1 Diabetes Prediction and Prevention Project (DIPP) found a correlation with CVB1, but also found reductions in risk with CVB3 and CVB6 (63). Thus, it is unclear if there are strain specific properties that may act to promote or reduce type 1 diabetes. Notably, the P2-C gene product of CVB4 shares sequence similarity to GAD65 at amino acids 249-279, which suggests a potential for antigen mimicry, whereby the immune system reacts against the foreign epitope and in turn also cross reacts with native antigen (64-66). However, experiments in NOD mice suggest that CBV4 promotes type 1 diabetes through a ‘bystander damage’ mechanism (67) rather than antigen mimicry (68; 69), where the infection causes a pro-inflammatory environment and allows sequestered antigen to be exposed to the immune system, similar to treatment with streptozotocin (70). These experiments also suggest that CBV4 infection is exacerbatory in nature, and still requires the presence of an already established autoreactive component to the immune system for development of the disease (71). In a similar vein, the herpes virus family, and in particular EBV and CMV, have also been implicated in increasing the risk of developing type 1 diabetes, although the evidence to suggest this is not as comprehensive as that of enteroviruses. Sequence homology of viral proteins from this family with GAD65, a native β cell antigen, have been suggested to be a trigger for type 1 diabetes (72). Notably, a case report found strong correlations between the detection of CMV and islet autoantibodies (73). These
viruses, along with many others, constitute a significant field of study in determining the casual factors around type 1 diabetes (55).

It has long been established in the NOD mouse model that environmental conditions greatly affect the relative proportion of diabetes incidence (74; 75). In addition to viral exposure discussed previously, bacteria may be an important factor in determining whether an individual goes on to develop type 1 diabetes. By and large, it is accepted that NOD mice housed in germ-free environments increases the incidence of diabetes (74). This is primarily attributed to alterations in intestinal microbiota as a result of environmental exposure, with antibiotic administration achieving similar results in some cases, although literature has found both pro- and anti-diabetic effects of antibiotics depending on the drug (74; 76). This is interesting, as it suggests that certain bacteria promote or inhibit autoimmunity and can be an exploitable avenue for therapeutics. Work investigating MYD88, a factor important in toll-like receptor (TLR) signaling, found that Myd88-null NOD mice were protected from diabetes (77). TLRs are important in the context of innate immunity, where they serve to recognize evolutionarily conserved antigens of bacteria to provide a ‘first line’ defense for the host. Adoptive transfer of CD4+ T cells from BDC2.5 mice, carrying a transgenic TCR to islet antigens and are normally diabetogenic, did not expand in the lymph nodes of Myd88-null NOD mice (77). However, housing Myd88-null NOD mice in germ free conditions with antibiotics restored the development of type 1 diabetes (77). Collectively, these results suggest a dependency of select species within the microflora that promote a tolerogenic or non-tolerogenic environment.

Other factors have been correlated with type 1 diabetes development, but the relative risk these factors induce is still debated (24). Substitution of cow’s milk versus infant breastfeeding early in life has been proposed to promote immune reactivity to the islet because of serum
antibodies that cross-react with milk albumin and ICA1 (p69), a β cell surface protein (78). Follow up studies have been inconclusive, and this hypothesis is generally considered to be minor relative to other suggested causal factors. Similarly, wheat proteins and vitamin D deficiency have also been suggested to contribute to type 1 diabetes development (24). With respect to Vitamin D, high throughput screening work from our lab has found that calciferol and cholecalciferol protects β cells from cytokine induced apoptosis, suggesting that amelioration of diabetes could stem in part from direct protection of pancreatic β cells rather than effects on the immune system (79). Although many studies have shown correlation with type 1 diabetes, mechanistic insights behind these factors are still unclear.

1.1.8. Progression of Type 1 Diabetes

Common to all the proposed causes of type 1 diabetes is the underlying concept of autoimmunity. Upon an unknown trigger, professional antigen presenting cells (APCs) like macrophages and dendritic cells (DCs) phagocytose self-antigens and present them to naive autoreactive T cells in the pancreatic lymph node for activation (80). Interestingly, studies have shown that even physiological β cell death associated with tissue remodeling is sufficient to induce autoreactivity mediated by dendritic cells (81). The activation of T cells by APCs allows the T cell to infiltrate the islet alongside a host of other immune cells, also known as insulitis, leading to profound inflammation and cellular destruction in what would otherwise be a “T cell free” environment (80). Both CD8+ cytotoxic T and CD4+ helper T cells are involved in the development of type 1 diabetes, and the adoptive transfer of either cell type can initiate its development (82; 83).
Many different factors come together in the progression of type 1 diabetes with the eventual result of pancreatic β cell destruction. Fortunately, pancreatic β cells maintain high reserves of insulin stored in crystalline granules within the cell and compensation for the loss of β cell mass is effective to a certain extent (84; 85). It is estimated that 70-90% of β cell mass must be lost prior to the development of persistent hyperglycemia (6). This presents a problem, as our current method of definitive diagnosis of type 1 diabetes occurs at a stage where 70-90% of β cells are destroyed. This is illustrated in the model provided by Dr. George Eisenbarth, which illustrates relative β cell area over the progression of the disease (86). Upon triggering events compounded with various susceptibility factors, β cell mass is progressively lost (6). At this point in time, prognostic factors of disease such as the presence of islet autoantibodies are detectable in the serum of patients (6). As the disease progresses, loss of first phase insulin secretion in response to large bolus glucose challenge is also apparent, followed quickly by chronic glucose intolerance and a significant loss of circulating C-peptide (6). Unless patients are suspected to be pre-disposed to type 1 diabetes (i.e. through hereditary history), diagnosis of the disease tends to occur very late into its progression, where the majority of β cells are already destroyed (6). Interestingly, it is believed that a small population of β cells continue to survive and function within the pancreas of patients with type 1 diabetes, as evidenced by detectable C-peptide after the onset of the disease (87; 88). Furthermore, results from the DiViD study found that pancreatic islets were isolatable from diabetic organ donors, and restoration of first phase insulin secretion was achieved after culture in vitro (89). One phenomenon observed in human patients is the “honeymoon phase” in which partial remission is achieved through the transient use of exogenous insulin (90), and insulin use can be reduced or eliminated for months to years post diagnosis (91; 92). The leading hypothesis of this phenomenon is the relief of residual β cells from the stress of producing insulin and allows
a recovery of β cell function (24). Thus, it is clear that a thorough understanding of the processes behind type 1 diabetes will allow us to better target key pathways when developing treatment for the disease.

1.1.9. Contributions of Other Immune Cells to Type 1 Diabetes

Type 1 diabetes is believed to primarily be a T cell mediated disease, although contributions of the innate immune system also play a role in exacerbating inflammation. Natural killer (NK) cells are a part of the innate immune system and function as first line anti-tumour and anti-viral defense. In various mouse models of type 1 diabetes, NK cells extracted from the pancreas express greater markers of activation and degranulation, proliferate more rapidly, and produce greater levels of IFN-γ (93; 94). Depletion of NK cells within animal mouse models also seems to prevent the development of type 1 diabetes (95; 96), as well as the blockade of known activatory receptors like NKG2D and NKp46 ameliorates the disease (94; 97). Counter points for NK cells argue that they are protective against type 1 diabetes, where they seem to destroy antigen-presenting DCs (98). Furthermore, the involvement of other cell types such as the invariant natural killer T (iNKT) cell and its ability to promote both tolerogenic and inflammatory environments makes it difficult to elucidate concrete mechanisms of type 1 diabetes (99).

1.1.10. Prognostic and Diagnostic Measures of Type 1 Diabetes

Diabetes is formally diagnosed based on the inability to adequately control blood glucose as measured via an oral glucose tolerance test (OGTT) (100). Outside of a glucose challenge, glycemic levels are relatively volatile and easily manipulatable, especially for a patient with diabetes, so the assessment of adherence to medications by health care providers cannot be done
with blood glucose. An alternative biomarker of blood sugar is the level of glycated hemoglobin (HbA1c), which is correlated to the mean blood glucose levels over a period of weeks (101). Although HbA1c provides an alternative measure of glycemia, variations in baseline levels can occur from hemoglobinopathies or measurement in different ethnic backgrounds (100; 102). Therefore, clinicians will typically utilize several methods of measuring blood sugar to ensure that the readings are accurate.

Lack of reliable biomarkers makes it incredibly difficult to predict with certainty whether an individual will go on to develop type 1 diabetes. Serum islet autoantibodies are the most commonly used predictive factor, where they target antigens that are made in the islet. The four main autoantigens are: insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated antigen-2 (IA-2), and zinc transporter 8 (ZnT8) (24). Less prolific autoantigens include proteins like heat shock proteins (HSPs) and islet-specific glucose-6-phosphatase (IGRP) (24). Insulin autoantibodies are usually the first to be detected, where the second most prevalent autoantibodies are against GAD65 (103). As of today, assays for the presence of IAA, GADA, and IA-2A are clinically available, with ZnT8A use restricted to experimental research (24). Expression of a single autoantibody did not correlate strongly with diabetes development as found in the BABYDIAB study (104). The presence of multiple autoantibodies is usually indicative of prolonged expression rather than transient, and is strongly linked to the eventual development of type 1 diabetes (105; 106). Thus, a combination of OGTTs, serum autoantibodies, C-peptide, and HbA1c are all used regularly to profile development and progression of type 1 diabetes (100). Additional genetic testing may be conducted if the patient is suspected to have monogenic diabetes.

Circulating microRNAs (miRNA/miR) offer another potential avenue of type 1 diabetes diagnosis, and recent efforts have been made to identify miRNAs or series of miRNAs predictive
of disease development and progression. miR-375 has been studied extensively, due to its high expression specifically in the islet (107). It has been shown to suppress GSIS (107), and subsequent experiments in pre-diabetic NOD or streptozotocin treated mice increased miR-375 circulating in serum (108). Interesting, the opposite was true in sera of type 1 diabetes patients at the onset of disease, where miR-375 was found to be lower as compared to controls (109). This discrepancy could be attributable to its function in acting as a suppressor of GSIS, where the destruction of pancreatic β cells in type 1 diabetes patients occurs over a greater period of time as compared to animal models. This delay in destruction allows pancreatic β cells to downregulate the expression of miR-375, to allow surviving β cells to compensate for a loss in insulin production capacity. Similarly, numerous other miRNA species have been identified in the islet as well as circulating peripheral blood mononuclear cells (PBMCs), offering us an alternative to the use of autoantibodies for prognostic and diagnostic endeavours (110; 111).

1.1.11. Current Treatment Strategies for Type 1 Diabetes

Current strategies in the treatment of type 1 diabetes are focused on the management of blood glucose via exogenous replacement of insulin. Although many different strategies have been proposed to address the root of the disease, no viable methods have been developed that supersede the original strategy of insulin injection. Today, many different insulin preparations and modifications to increase activity and vary half-life are available (112). Similarly, detection of minute-to-minute glycemia has been greatly improved by advancements in continuous glucose monitoring (CGM) (113). Although the combination of CGM with automated insulin pumps in ‘closed-loop systems’ are touted as a promising area of development (114), no technologies currently maintain glucose homeostasis as well as endogenous pancreatic β cells.
Many different approaches have been suggested to tackle type 1 diabetes. Because type 1 diabetes is traditionally thought to be solely an autoimmune disease, clinical trials that have primarily focused on inhibiting the immune system to preserve residual β cell mass or to prevent the development of the disease. Some of the immune-targeted strategies include: broad spectrum immunosuppression, T cell specific immunosuppression, anti-inflammatories, antigenic induction of tolerance, transplantation of immunosuppressive cells, induction of Treg proliferation and activity, as well as combination therapy with several of the aforementioned strategies (115). Although preliminary results from these trials are promising, no clinical trial has yet provided us with a concrete method of halting, or even robustly slowing, the progression of type 1 diabetes (115). Current efforts are focused on the early diagnosis of type 1 diabetes with the development of better diagnostic criteria and available biomarkers to allow immunotherapy to preserve as much residual β cell mass as possible (115).

Islet transplantation is a method of restoring functional β cell mass available to patients with poorly controlled diabetes and are prone to hypoglycemic episodes (116). Although the idea of islet transplantation preceded the Edmonton Protocol developed in 1999, this protocol popularized islet transplantation as an effective and reliable means of restoring glucose homeostasis, with reduced or even eliminated requirements for exogenous insulin (116). Pancreatic islets are isolated from one or multiple cadaveric donor pancreata with the enzyme cocktail Liberase and transplanted into the hepatic portal vein, where patients were subsequently placed on a steroid-free immunosuppressive drug cocktail (117). Of the seven patients reported in the original study, all of them remained insulin-independent as compared to 8.2% of patients treated with previous protocols one year post-transplantation (117). Although islet transplantation has been highly successful in restoring glucose homeostasis in patients, follow up studies have
shown that grafts are prone to failure within several years after transplantation (118). Unfortunately, the lack of available donors, recurrent autoimmunity, and the maintenance of viability during extraction, processing, and transportation of the islet grafts have limited the ability of this technique to be a ubiquitous technique for all patients (119).

The replacement of pancreatic β cells into patients with type 1 diabetes is theoretically a feasible and efficacious way of restoring glucose homeostasis, as demonstrated by the transient success in cadaveric islet transplantation. Work with stem cell differentiation has greatly increased the excitement around the possibility of a steady pool of transplantable material. However, there is no viable protocol to date that generates fully functional pancreatic β cells in vitro (120). Although stem-to-β cell differentiation protocols have progressed remarkably to the point of transient amelioration of hyperglycemia in β cell deficient mice, proper signatures of β cell function (e.g. appropriate calcium signaling) have yet to be fully emulated (120-122). Specifically, in vitro derived β-like cells have only modest insulin secretion capacity as compared to primary β cells, which speaks to the need for additional modifications to the differentiation protocol (121). Similar to cadaveric islet transplantation, viability of the graft remains a key area of concern (123). Examples of encapsulated β cells to create an ‘immune privileged’ environment has seen promising preliminary results in mice (124; 125), but results from clinical trials in humans have not yet been reported. Finally, concerns of tumour formation from the transplantation of multipotent cells will need to be addressed (123). In summary, the treatment strategy for type 1 diabetes in 2017, consisting of exogenous insulin replacement for most people, is not conceptually different from that employed almost 100 years ago. It is clear that a better understanding of type 1 diabetes and new approaches are required to make progress in this field.
1.2. Physiology and Pathophysiology of the Pancreatic β Cell

1.2.1. Pancreatic β Cell Death in Type 1 Diabetes

Pancreatic β cell death in type 1 diabetes is believed to be mediated by selective autoimmune destruction. Cell death occurs in many ways, such as direct contact with macrophages and cytotoxic T cells and via the surrounding inflammatory cytokines (126). Notably, primary β cells in vitro can undergo a variety of different pathways to cell death, including necrosis, with only 50% of them undergoing “classical” apoptosis when challenged with an inflammatory cocktail of cytokines (IFN-γ, IL-1β, TNF-α) (127).

Cytotoxic T cells kill β cells directly after recognition of their cognate antigen by the respective TCR through perforin pore formation and granzyme protease activity (128). Perforin deficient mice backcrossed onto the NOD background have significantly reduced diabetes incidence, as well as a delayed age of diabetes development (129). Although perforin deficient, suppressor of cytokine signalling 1 (SOCS-1) overexpressing NOD mice was also largely protected from diabetes, a small subset continued to develop the disease, which suggests alternative mechanisms of β cell death apart ‘traditional’ mediators of β cell death (e.g. perforin, inflammatory cytokines, and FasL) (130). In contrast, studies in perforin deficient NOD8.3 TCR mice that produce T cells with a transgenic TCR specific to β cells revealed that β cells were exclusively destroyed by Fas and diabetes incidence was increased when perforin was absent (131). A mutation in fas (fas<sup>lpr</sup>) also prevented the development of diabetes in NOD mice (132). Intriguingly, subsequent studies of NOD mice homozygous for the fas<sup>lpr</sup> allele (NOD<sup>lpr/lpr</sup>) found that insulitis was inhibited (133), and transplantation of isolated islets into diabetic recipients did not protect them from autoimmune destruction (134; 135). Taken together, perforin, granzyme and
FasL are largely responsible for direct T cell mediated cytotoxicity, although other factors play an important role in promoting the progression of disease.

IL-1β, IFN-γ, and TNF-α constitute the standard cocktail with which scientists perform β cell killing assays *in vitro*. Treatment of β cells with either IFN-γ or IL-1β alone does not induce robust death in some studies, indicating that both factors are required for appreciable cytotoxicity (126). IFN-γ acts on β cells via cell surface tyrosine kinases JAK1 and 2, leading to the activation of the transcription factor STAT1, which is believed to be the point of synergy with IL-1β signalling for cell death (126). Endogenous inhibitory mechanisms of cytokine signalling are also present in the cell, and are mediated through SHP and SOCS (126). Knockout of STAT1 inhibits cytokine induced β cell death (136), consistent with literature reporting protective effects of SOCS-1 and SOCS-3 overexpression (137). IL-1β activates the NF-κB pathway in both rodent (138) and human (139) islet cells, where the inhibition of NF-κB activation with transient infection of an adenovirus containing an NF-κB super-repressor lowered the amount of IL-1β/ IFN-γ induced (138) and FasL induced β cell death (139). NF-κB was shown to increase the expression of iNOS in rat β cells (140), which increases the local production of NO as demonstrated in INS-1E cells treated with IL-1β and IFN-γ (141). Of 698 genes identified by microarray when INS-1E cells were treated with IL-1β and IFN-γ, approximately 321 of them, were found to be dependent on NO signalling, suggesting a significant portion of NF-κB effect on the β cell is mediated via NO (141). Amongst a multitude of effects, NF-κB was found to decrease transcription factors influencing β cell identity (e.g. Pdx1 and Isl1) (142). Reduction of Pdx1 in mice is sufficient alone to increase β cell apoptosis and induces ER stress through the reduction of SERCA2 (142-144). Loss of *Pdx1* depletes the ER of its calcium store, triggering ER stress and the expression of *Gadd153/CHOP* in a mechanism similar to the SERCA inhibitor thapsigargin (145; 146). ER stress
contributes to the dysfunction of pancreatic β cells, and markers of ER stress were found in islets from patients with type 1 diabetes (147) and NOD mice prior to the onset of diabetes (148). Interestingly, in vivo inhibition of NF-κB seems to also inhibit GSIS in mice, which suggests detrimental effects of NF-κB inhibition either in the long term or during development (126; 149). Thus, the exposure of pancreatic β cells to inflammatory cytokines promotes increased susceptibility to T cell killing and direct induction of apoptosis.

1.2.2. β Cell Proliferation and Transdifferentiation

One area of considerable interest in the treatment of type 1 diabetes is the development of viable methods to induce β cell proliferation endogenously to replace cellular mass and insulin production capacity. Lineage tracing studies have shown that the any change in β cell mass because of natural cellular turnover or injury is replenished through self-renewal rather than stem cells (150-152). Pancreatic β cells are notorious for their low proliferation rates (152; 153), especially during adulthood (154; 155). Although many different compounds and endogenous factors have been proposed to induce β cell proliferation (156-159), none have yet to be proven effective in a clinical trial.

In a similar vein, the conversion of other cells into glucose responsive, insulin producing β cells is a field of considerable interest. Several studies have shown the possibility of conversion either by lineage tracing or manipulation from other cell types (e.g. exocrine, ductal, islet alpha) into insulin positive cells (160-162). These studies often utilize severe pancreatic injury and/or chemical compounds to promote the expression of transcription factors that guide the cell down a developmental path. Thus, they remain an important area of study, as β cells become ‘de-differentiated’ as a result of injury and/or exhaustion.
1.2.3. Concept of β Cell ‘Fragility’ and β Cell Targeted Therapies

In addition to autoimmune pressures on pancreatic β cells, a recent article published by Dooley et al. (2016) suggests that type 1 and 2 diabetes can be associated with a phenomenon known as β cell ‘fragility’. Male NOD mice with the Idd1 susceptibility locus replaced (NOD₅) to prevent autoimmunity and hemizygous for hen egg lysozyme (HEL) expression driven under the Ins2 promoter developed spontaneous diabetes as compared to wild-type C57BL/10 mice also expressing HEL under the Ins2 promoter (163). The lack of autoimmunity in this induction of diabetes was further confirmed through the interruption of T and B cell development via additional crosses with the Prkdc<sup>scid</sup> and Rag1<sup>−/−</sup> mouse lines (163). Diabetes development was primarily attributed to the induction of ER stress and the unfolded protein response (UPR) within the pancreatic β cells, leading to aberrant insulin processing and subsequent glucose intolerance (163). Notably, Xrcc4, a DNA repair protein, was associated with the development of diabetes in these mice, where it has been previously shown that the knockout of Xrcc4’s binding partner, Lig4, results in diabetes due to impaired DNA repair leading to senescence (164; 165). Also identified was Gli3, which was previously reported to be required for INS-1 β cell survival (166). The Dooley et al. (2016) study highlights the ability of genetic mutations to increase the risk of β cell death independent of autoimmune contributions. Although it is well known that the vast majority of pancreatic β cells are destroyed via autoimmunity, residual β cells that overexpress insulin as a compensatory mechanism for metabolic dysfunction lose their ability to proliferate (167) and are prone to apoptosis induced by ‘second hit’ stressors (163). Collectively, these studies support the possibility of β cell-centric approaches to type 1 diabetes treatment. Targeting β cell health may strengthen their ability to resist apoptotic signals as an alternative or adjunct to immunosuppression.
1.3. Overview of Pancreatic β Cell Ion Channels

1.3.1. β Cell Excitability and the Classical Insulin Secretion Pathway

The pancreatic β cell is electrically excitable, exhibiting membrane potential oscillations in the presence of glucose (168). At low glucose concentrations, β cells remain electrically silent and rests at a membrane potential of -60 mV to – 70 mV (168; 169). The low resting membrane potential is primarily attributed to the ATP-sensitive potassium channel that is open at low levels of ATP relative to ADP, hyperpolarizing the cell (170). The cascade of events leading to insulin secretion starts with the entry glucose into the pancreatic β cell via GLUT2 in rodents or GLUT1 in humans (171). Processing glucose through glycolysis and then the TCA pathway eventually yields the formation of ATP, shifting the ratio between intracellular ATP and ADP (170). This ratio shift closes the K_{ATP} channel, subsequently eliminating the efflux of potassium and depolarizing the membrane (170). As the membrane depolarizes, Na_{v} channels and T-type calcium channels contribute to the depolarization, which then triggers Ca_{v} channels (e.g. P/Q, N, and L) to depolarize at -20 mV, representing the commitment step to insulin secretion via the massive influx of calcium. The increase in intracellular calcium activates exocytotic machinery, called SNARE proteins, that dock and fuse the insulin vesicles to the plasma membrane (172). Delayed rectifier voltage dependent potassium channels then repolarize the membrane to return the cell to basal potentials (173). This terminates the insulin secretion pathway, returning the cell to basal state where it can then receive additional stimuli for subsequent secretion.

1.3.2. Voltage Gated Sodium Channels in β Cells

As compared to many ion channels within the pancreatic β cell, functions of voltage gated sodium channels (VGSCs) has been understudied. This is, in part, due to the fact that they play a
more prominent role in human β cells (174; 175), rather than mouse β cells that are more readily available for study. Structurally, VGSCs are comprised of six alpha helices, with the S4 segment acting as a voltage sensor and S5-S6 segments forming the pore of the channel (176; 177). VGSCs functionally exist as a homotetramer, where four identical alpha subunits come together to create a channel (176). Nine distinct variants of the alpha subunits are present within the VGSC gene family (Nav1.1-1.9 or Scn1a-Scn9a) (178). Nav1.7 (Scn9a) is most highly expressed in neuronal tissue involved in pain (179), but other tissues have also been shown to express VGSCs (180-182). Our lab and others have shown that Nav1.3 and Nav1.7 are the most abundantly expressed in rodent β cells (79; 169; 180). Other cells of the endocrine pancreas (α and δ cells) also express VGSCs, where they are known to affect excitability and function (180; 181). Additionally, VGSCs possess a β subunit that interacts with the α subunit homotetramer to regulate trafficking and channel behaviour (169). Scn1b is the most abundant of the Scn1b-Scn4b family, and allows the channel complex to inactivate after prolonged patency (169).

Initial studies of VGSCs in rodent cells found discrepancies in the ability of the general VGSC inhibitor tetrodotoxin’s (TTX) to inhibit inward sodium transients (183; 184). It was later discovered that β cells isolated from NMRI mice possessed a TTX-sensitive sodium current, although this current had a half maximal steady state inactivation of -100 mV (185). This was confirmed, as changes in membrane potential were not observed with addition of TTX from 3 mM to 20 mM glucose (185). This suggested that insulin secretion in murine β cells was unlikely to rely on VGSC, as the resting membrane potential of pancreatic β cells is approximately -70 mV where the VGSCs would be already inactivated. Experiments with rat β cells found a half maximal steady state inactivation voltage of -40 mV, well within the range of resting membrane potential (186). Rats, along with dogs, pigs, and humans have larger contributions of VGSCs to membrane
potential, as all of these species exhibited a decrease in excitability with TTX treatment (174; 186; 187). Although VGSCs in mice seem to play a relatively minor role, knockout of Scn1b subunit impairs GSIS and results in glucose intolerance (188).

Work with murine β cells found that the knockout of Scn3a resulted in inhibition of GSIS as compared to Scn9a, even though Scn9a is the most expressed VGSC family member (169). Scn3a was also found to be important in approximately 30% of β cells assayed, providing evidence of functional heterogeneity, which may account for the excitable β cell hubs that propagate electrical signals throughout the islet (169). Biophysical analysis of Scn9a found its half maximal steady state inactivation voltage to be -105 mV (169). This finding suggests that Scn9a does not contribute significantly to β cell insulin secretion. As discussed in later sections however, administration of use-dependent sodium channel blockers like carbamazepine and lidocaine as opposed to use-independent blockers like TTX can modulate long-term pancreatic β function and survival (79; 189).

1.4. Background on Carbamazepine

1.4.1. Clinical Use of Carbamazepine

Carbamazepine is clinically used to treat acute manic episodes, bipolar disorder, and epilepsy (190). Carbamazepine is a ‘narrow’ type anti-epileptic (191), where broad action antiepileptics also inhibit thalamic ‘spike and wave’ discharges of calcium channels believed to be important in absence seizures (192). Carbamazepine’s primary effect is inhibiting voltage gated sodium channels in a use-dependent manner (190). This ‘use-dependence’ refers to the inhibition of channels only after they have been activated, as compared to ‘use-independent’ blockers that are independent of channel activity. Clinically, it is best used for partial and generalized tonic-
clonic seizures, but may exacerbate absence seizures. Problematic features of carbamazepine are slow and erratic absorption, high affinity for protein (~75% bound to protein), and increases the activity of cytochrome P450 enzymes (which makes it an additional consideration in combination with other therapeutics) (190). Half life of carbamazepine after a single administration is approximately 35 hours, although this number decreases with repeated dosing likely due to the self-induction of metabolic enzymes that inactive the drug (190).

1.4.2. Carbamazepine’s Influence on β Cell Survival and Function

The Johnson lab previously conducted a high-throughput, multi-parameter, image-based screen to identify FDA-approved drugs from the Prestwick compound library that protect β cells against a cocktail of inflammatory cytokines (TNF-α, IFN-γ, IL-1β) designed to mimic type 1 diabetes conditions (79). Carbamazepine, a use-dependent sodium channel blocker that is used clinically as an anti-epileptic, was identified as a compound that reduced β cell death in this context (79). The protective effect on β cells against toxic cytokines was seen with two use-dependent sodium channel blockers, carbamazepine and lidocaine, but not the use-independent sodium blocker tetrodotoxin (79). Another screen from our group found that carbamazepine promoted insulin production (189) and other sodium channel blockers have been found by other groups to promote β cell function in reporter-based compound screens (193). Based on these findings, we concluded that carbamazepine merited additional study in an in vivo model of type 1 diabetes.

Carbamazepine blocks inward sodium currents in pancreatic β cells and directly acts on the Nav1.7 sodium channel isoform in a use-dependent manner (79; 189). We and others have found that Nav1.7 is the principal voltage-gated sodium channel alpha subunit isoform expressed in purified β cells (79; 169). Scn9a knockout mice, which lack the Nav1.7 current, have normal
insulin secretion but improved cellular insulin content with age (169; 189). We have previously shown that carbamazepine does not significantly inhibit insulin secretion from mouse islets (79), although preliminary data on two batches of human islets suggested the possibility of an inhibitory effect (Lee, unpublished data).

1.5. Overview of Research Objectives

In the present study, we investigated the effects of carbamazepine on type 1 diabetes incidence, β cell function and pancreatic pathology in the non-obese diabetic (NOD) mouse model. We identified a clear difference in diabetes incidence between control and carbamazepine treated animals, with positive effects on pancreatic β cell function. Peripheral immune cell composition and inflammation were largely unchanged in drug treated animals. Collectively, our data suggest carbamazepine protects non-obese diabetic mice from type 1 diabetes primarily via the promotion of β cell survival and function.
Chapter 2: Materials and Methods

2.1. Diabetes Tracking and In Vivo Physiology

Mice were housed in accordance with the University of British Columbia Animal Care Committee guidelines. Female, non-obese diabetic (NOD) mice were used for this study due to their higher incidence of spontaneous diabetes as compared to males. The oral carbamazepine dose we used was chosen based on a previous in vivo behavioural study (194). Mice were weaned at 4-6 weeks of age onto irradiated 0.5% w/w carbamazepine (Sigma, C4024) supplemented LabDiet 5053 (5BTY) or control LabDiet 5053 formulated by TestDiet®. A total of 42 mice were used to assess incidence of diabetes (‘incidence’ cohort) until 25 weeks of age. Mice were monitored weekly for body weight and 4-hour fasting blood glucose. Mice were considered diabetic at two consecutive measurements of fasting blood glucose above 16 mmol/L. Carbamazepine levels in serum from blood obtained by terminal cardiac puncture immediately after euthanization by CO₂ were quantified after 1:1000 dilution (in assay diluent) using a commercial ELISA (Abraxis, 515585) according to the manufacturers protocol. An age matched, pre-diabetic (‘chase’ cohort) of mice (n = 5 for both control and carbamazepine treated) was euthanized 7 weeks of age (prior to the time when overt hyperglycemia is seen in any group). Glucose tolerance was assessed in the pre-diabetic ‘chase’ cohort of NOD mice (6 weeks of age) injected with 2 g/kg glucose after a 4 hour fast. After resting for 1 week, a second injection of 2 g/kg glucose was given after a 4 hour fast and 5 μl of blood was collected for insulin quantification by a commercial insulin ELISA kit (Alpco, 80-INSMS-E01).

2.2. Histology, Immunofluorescence and Islet Infiltration Scoring

Pancreata were fixed in 10% formalin for 48 hours prior to storage in 70% ethanol at 4°C.
Paraffin embedded sections were prepared, sliced and stained with hematoxylin/eosin (H&E) or hematoxylin alone, with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) by WaxIT Histology Services Inc. (Vancouver, BC). Whole H&E-stained slides were imaged by WaxIT Histology Services at 20X magnification. Five sections separated by 100 μm were scored blindly for infiltration of mononuclear cells into islets according to previous protocols (195). TUNEL was imaged on a bright field Zeiss Axio Imager A1 microscope at 40X magnification, and the number of TUNEL-positive cells were normalized to the number of islets visible on each pancreatic section.

Three sections per mouse, separated by 100 μm, were deparaffinised, subjected to an antigen retrieval process, and stained according to a published protocol (167) for quantification of insulin positive β cell area. Insulin was stained with 1:250 guinea pig anti-insulin (Abcam, ab7842) and 1:1000 goat anti-guinea pig AlexaFluor 594 (Invitrogen, A-11076). Slides were subsequently counter-stained with 4’, 6-diamidine-2’-phenylindole dihydrochloride (DAPI) in VECTASHIELD HardSet Antifade Mounting Medium (Vector Laboratories, H-1500) and whole slides were imaged using a 10X (NA 0.3) objective on an ImageXpress MICRO robotic microscope and analyzed using MetaXpress 5.0 software (Molecular Devices). β cell area was quantified by normalizing insulin positive area to the respective section size as determined by DAPI staining and was reported as a ratio of the two numbers. A subset of slides were also stained with 1:500 rabbit anti-PCNA (Cell Signaling Technologies, D3H8P) and 1:1000 goat anti-rabbit AlexaFluor 488 (Invitrogen, A-11008) and subsequently imaged on a Zeiss Axiovert 100 microscope using a 40X (NA 0.6) objective. Images were subsequently processed in Slidebook 5.5 (Intelligent Imaging Innovations) and Fiji (ImageJ) software (196).
2.3. Flow Cytometry of Pancreatic Lymph Nodes

Pancreatic lymph nodes were isolated from 7 week-old pre-diabetic NOD mice and single cell suspensions were obtained by passing them through a 40 μm cell strainer. Lymphocytes were stained with antibodies specific for: CD3 (conjugated with e450, used at 1:200, excited by a 405 nm laser)(48-0033), CD4 (conjugated with FITC, used at 1:100, excited by a 488 nm laser)(11-0042), and CD8 (conjugated with APC780, used at 1:100, excited by a 633 nm laser)(47-0081), all from eBioscience. 5000 live events were collected on a LSRII flow cytometer in the UBC Life Sciences Institute Flow Core and results were analyzed on Flowjo X 10.0.7 software.

2.4. Multiplex Cytokine Assay

Mice were euthanized and cardiac puncture was performed to isolate whole blood upon diabetes development in the incidence cohort or at 7 weeks of age in the chase cohort. Blood was left to clot at room temperature for 15 minutes before centrifugation at 1000 times $g$ at 4°C. Samples were then stored at -20°C until analysis. A panel of 7 cytokines from the chase cohort and a subset of the incidence cohort were assayed using the Bio-Plex Pro™ Mouse Cytokine Th1 Panel (Bio-Rad, L6000004C6) according to the manufacturer’s instructions. Cytokine levels under the limit of threshold were assigned a value of zero when other analytes in the blood sample were detectable.

2.5. Statistics and Data Presentation

Unless indicated otherwise, all data are expressed as mean ± SEM. Statistical significance was defined at a threshold of $p < 0.05$. Diabetes incidence was measured by Kaplan-Meier analysis and quantified by log-rank test. Insulitis significance was quantified by Mann-Whitney U test with
a minimum count of 100 islets quantified per treatment group. All other single variable based
group comparisons were quantified by Student’s t-test using GraphPad Prism software.
Chapter 3: Results

3.1. Carbamazepine Reduces Diabetes Incidence in NOD Mice

Our group has previously shown that carbamazepine protects pancreatic β cells against a cocktail of pro-inflammatory cytokines in vitro (79). To test whether carbamazepine protects β cells in vivo, we utilized two cohorts of female, non-obese diabetic (NOD) mice: a diabetes ‘incidence’ cohort and a pre-diabetic and an age matched ‘chase’ cohort (Figure 3.1). In both cohorts, mice were weaned onto diet supplemented with or without carbamazepine and assessed weekly for fasting blood glucose.

We studied the ‘incidence’ cohort and recorded that 80% of the control mice developed diabetes by the age of 25 weeks, as evidenced by at least 2 fasting blood glucose measurements above 16 mM (Figure 3.2A). This is consistent with the diabetes incidence rate in NOD mice in our colony (195) and in other facilities (75). In contrast, diabetes was only observed in only 40% of the NOD mice treated with carbamazepine. Although differences in diabetes incidence diverged at about 16 weeks in age, we noted that carbamazepine treated mice had significantly lower fasting blood glucose even prior to chronic hyperglycemia at 6-10 weeks of age (Figure 3.2B). After euthanasia (either upon the discovery of chronic hyperglycemia or at 25 weeks of age), mean serum carbamazepine levels quantified by ELISA were 14.98 μM (Figure 3.2C). Notably, serum carbamazepine levels in individual mice were not correlated to the age of euthanasia (data not shown). Carbamazepine treated mice exhibited a significant decrease in body weight during the first week of administration (Figure 3.2D), and this decrease was persistent throughout the course of the study. Interestingly, this was not associated with a decrease in average food consumption (Figure 3.2E). Collectively, these data demonstrate that carbamazepine treatment at levels used in other studies (194), can prevent 50% of diabetes cases in our NOD mouse population within 25
weeks of age.

3.2. Carbamazepine Improves Glucose Tolerance in Pre-Diabetic NOD Mice

In order to identify the upstream mechanisms mediating the protection from diabetes in carbamazepine-treated NOD mice, and due to the variable age in which mice were euthanized within the ‘incidence’ cohort, we examined our ‘chase’ cohort of age-matched NOD mice sacrificed prior to the onset of hyperglycemia. Glucose tolerance at 6 weeks of age was significantly improved in carbamazepine treated mice compared to control animals (Figure 3.3A and B). Glucose stimulated insulin secretion normalized to basal secretion tended to be higher in a small study including some of the same mice at 7 weeks, although the insulin measurements were highly variable (Figure 3.3C). Collectively, these experiments show that β cell function is improved by carbamazepine rapidly after the initiation of treatment.

3.3. Pancreatic β Cell Survival Following Carbamazepine Treatment

To determine the effects of carbamazepine on β cell survival in vivo in the context of a fully competent immune system, and to test whether alterations in β cell mass could account for the improved glucose tolerance in our drug treated animals, we performed histological analyses and TUNEL staining. We found no significant differences in islet cell death as measured by TUNEL staining (Figure 3.4A) in the pre-diabetic ‘chase’ cohort of mice treated with carbamazepine as compared to control (Figure 3.4B). Although it should be noted that this technique is notoriously insensitive due to the transient (~3 hours) nature of TUNEL labelling in vivo where macrophages rapidly dispose of apoptotic β cells (197; 198). Therefore, we next tested whether physical β cell mass was altered in a robust way by carbamazepine treatment. We
observed no statistically significant differences in β cell area (Figure 3.4C) between treatments groups in the pre-diabetic ‘chase’ cohort (Figure 3.4D) nor the ‘incidence’ cohort (Figure 3.4E). Although it was noted that, while all mice had profound β cell loss with age and disease progression (when comparing the ‘incidence’ and ‘chase’ cohorts), a subset of mice in both treatment groups in the ‘incidence’ cohort maintained a greater β cell area as compared to their littermates, exhibiting a bimodal β cell area distribution. Carbamazepine treated mice had approximately double the number of ‘high β cell area’ animals as compared to controls, correlating well with initial findings of diabetes incidence. Together, these data hint that β cell preservation may play a role in the protective effects of carbamazepine, but that a much better powered study would be required to overcome the heterogeneity of disease incidence in the NOD model.

We also assessed whether carbamazepine had the ability to stimulate β cell proliferation in vivo by staining some sections for proliferating cell nuclear antigen (PCNA) (Figure 3.4F). We found no compelling instances of PCNA staining in any insulin-positive cells, suggesting that marginal differences, if any, in β cell area were not attributable to proliferative effects of carbamazepine. However, infiltrating mononuclear cells surrounding the islet in both treatment groups showed robust proliferation, demonstrating that the staining protocol was capable of identifying proliferating cells.

3.4. Carbamazepine Reduces Insulitis in NOD Mice

Having observed no robust differences in proliferative capacity of the infiltrating cells surrounding the islets, we looked to assess other markers of immune function to determine if carbamazepine had any significant immunosuppressive abilities. Immune-islet infiltration (insulitis) in H&E-stained pancreatic sections was quantified and scored according to standard
criteria (195) (Figure 3.5A). At 7 weeks of age, carbamazepine treated NOD mice had an overall decrease in insulitis as compared with age-matched controls. In particular, we counted more islets with no insulitis and fewer islets with peri-insulitis (Figure 3.5 B). These results indicate that carbamazepine may, directly or indirectly, modulate the immune cell attraction to islets or infiltration into islets.

3.5. T Cell Populations and Circulating Markers of Inflammation

We next sought to perform experiments to assess peripheral T cell populations and systemic inflammation in animals treated with or without carbamazepine. We isolated lymphocytes from pancreatic lymph nodes from our ‘chase’ cohort and stained for markers of T cell identity. Flow cytometric analyses for CD3+ cells revealed no significant differences in proportions of CD3+CD4+ or CD3+CD8+ T cells (Figure 3.6A and B). Stimulated lymphocytes were also assessed for exocytotic activity via in vitro incubation with anti-CD107a antibody stimulated by PMA and ionomycin in CD4+ and CD8+ populations (Figure 3.6C and D). However, it was observed that stimulation of the lymphocytes with PMA and ionomycin failed to induce exocytotic activity, and no detectable differences between control and carbamazepine-treated mice were observed.

We also examined a panel of seven cytokines present in serum of the ‘chase’ (Figure 3.7 A-F) and ‘incidence’ cohorts (Figure 3.7 G-L) as a measure of whole animal inflammatory status. Of the 7 analytes we examined, we were unable to detect IL-2 within the standard range of the assay and we therefore excluded it from further analyses. Pre-diabetic, carbamazepine treated mice (‘chase’ cohort) had significantly lower circulating IL-1β as compared to controls, but this difference was not seen in older ‘incidence’ cohort. There were no differences in circulating
concentrations of IFNγ, IL-12, IL-6, TNF-α, or IL-10 in either the 7-week pre-diabetic cohort or in the incidence cohort studied up to 25 weeks of age, although many of the data distributions were bimodal in the old mice, as would be expected given the heterogeneity of disease incidence (Figure 3.7 A-L). Although islet specific immune cells were not examined, these findings are consistent with the idea that carbamazepine prevents or delays type 1 diabetes by via mechanisms that do not involve an obvious change in immune system activation based on most of the parameters we measured. The improved β cell function may explain the reduced incidence of immune cell infiltration into the islets.
‘Incidence’ cohort
Non-age matched, diabetic

Control: 20 mice
Carbamazepine-treated: 22 mice

Weeks of age

‘Chase’ cohort
Age matched, non-diabetic

Control: 5 mice
Carbamazepine-treated: 5 mice

Weeks of age

Figure 3.1 Study design. Schematic diagram of experiments conducted on non-obese diabetic mice (non-age matched – ‘incidence cohort’) or a pre-diabetic, age-matched (‘chase’ cohort).
Figure 3.2 Oral carbamazepine ameliorates diabetes incidence NOD mice. Female NOD mice were weaned onto LabDiet 5053 supplemented with 0.5% w/w carbamazepine (n = 22) or LabDiet 5053 alone (n = 20). Mice were euthanized upon reaching diabetic status (2 consecutive weekly measurements of fasting blood glucose > 16.0 mmol/l) and diabetes incidence was depicted via a Kaplan-Meier curve (A) with its corresponding fasting blood glucose (B). Upon euthanization, serum was collected via cardiac puncture and serum carbamazepine was quantified by ELISA (C). Body weight (D) and daily food consumption (E) averaged over the week was also collected.
**Figure 3.3 Improved glucose homeostasis in pre-diabetic NOD mice.** Female NOD mice were either fed LabDiet 5053 supplemented with 0.5% w/w carbamazepine (n = 5) or LabDiet 5053 alone (n = 4). 6 week old mice were challenged with an intraperitoneal injection of D-glucose (2 g/kg) and blood glucose was assessed over a period of 2 hours (A) with corresponding area under the curve (AUC) quantification (B). One week later at 7 weeks of age, mice were challenged again with 2 g/kg glucose and glucose stimulated insulin secretion (normalized to baseline) was quantified over a 15 minute period (C).
Figure 3.4 Histological assessment of β cell apoptosis, β cell proliferation and β cell area. Apoptotic cell death was quantified via TUNEL staining (A) in the pre-diabetic ‘chase’ cohort (B). β cell area was quantified via insulin staining (C) and normalized to total pancreatic area in the pre-diabetic ‘chase’ cohort (D) and the diabetic ‘incidence’ cohort (E). Mice from the pre-diabetic ‘chase’ cohort were also stained with co-stained for PCNA and insulin (F). Scale bars given at 10 μm.
Figure 3.5 Carbamazepine treatment reduces insulitis in NOD mice. Insulitis in the carbamazepine-treated and control animals from the pre-diabetic ‘chase’ cohort was blindly scored in by H&E stained pancreata based on standard criteria, with examples shown in (A), and quantified (B). Scale bars given at 100 μm.
Figure 3.6 Analysis of pancreatic lymph node immune cell populations in carbamazepine-treated and untreated NOD mice. Mice from the pre-diabetic ‘chase’ cohort were euthanized and lymphocytes were isolated from the pancreatic lymph node. Cells were subjected to surface staining with CD3, CD4, and CD8 antibodies. CD3+ cells were gated and proportions of CD4+/CD8+ cells were analyzed (A) and depicted in a bar graph (B). CD4+ (C) and CD8+ (D) T cells were also stained for CD107a and representative plots are provided.
Figure 3.7 Circulating markers of inflammation in carbamazepine-treated and untreated NOD mice. Serum collected at experimental endpoint was assessed via a 7-plex mouse Th1 inflammatory Luminex kit for the pre-diabetic ‘chase’ cohort (A-F) and a subset of the ‘incidence’ cohort (G-L).
Chapter 4: Discussion

4.1. Carbamazepine Reduces the Incidence of Type 1 Diabetes in NOD Mice

The goal of the study was to investigate whether carbamazepine’s protective effects in vitro were translatable to an in vivo autoimmune model of diabetes. Specifically, we used the NOD mouse model of type 1 diabetes to determine the effects of this drug and performed studies to assess its likely mode of action. As we predicted by our previous in vitro screens and follow-up experiments in which we observed cellular-level protection and improved \( \beta \) cell function (79; 189), we found a marked decrease of diabetes incidence in mice fed a diet that included carbamazepine at clinically-relevant concentrations. All surviving mice in the incidence cohort were euthanized at 25 weeks of age to prioritize histological experiments, therefore we were unable to conclude whether carbamazepine confers protection past this time point. Although we did not identify the precise cellular and molecular mechanisms that reduced diabetes incidence in carbamazepine treated mice, we did observe a striking improvement in glucose tolerance in carbamazepine treated animals even prior to the onset the first case of diabetes, suggesting that the primary cellular target of this drug is pancreatic \( \beta \) cell function and/or survival.

We were unable to directly demonstrate a robust difference in in vivo \( \beta \) cell survival or \( \beta \) cell mass between the control and treatment groups, although there was a trend towards increased \( \beta \) cell area was observed within the incidence cohort. Although the majority of mice experienced dramatic reduction in \( \beta \) cell area in the incidence cohort as compared with the chase cohort, a bimodal distribution was observed with double the number of mice exhibiting higher levels of \( \beta \) cell mass in the carbamazepine treated group, confounding analysis. Similarly, trend towards an increase in glucose stimulated insulin secretion was also observed in carbamazepine treated mice, but the variability in the data resulted in inconclusive findings. Nevertheless, the observation that
carbamazepine dramatically reduced type 1 diabetes by 25 weeks of age in NOD mice demonstrates the potential of the compound and its underlying mechanism in targeted treatment of the disease.

It is also possible that the dose of carbamazepine achieved in vivo was not sufficient for anti-apoptotic effects in the face of full autoimmunity. It should be noted that 10 μM carbamazepine was not completely protective in our in vitro via high throughput screen, and 100 μM carbamazepine was required to achieve a complete block of cytokine-induced β cell death in our lab’s previous work (79). By comparison, carbamazepine increased insulin mRNA and inhibited the Nav1.7 sodium channel at concentrations as low as 0.01 μM (189). Serum levels of carbamazepine in our study was estimated to be approximately 15 μM, but it is unknown what concentrations were available to the pancreatic β cell. Thus, it is plausible that carbamazepine primarily protects NOD mice from type 1 diabetes by improving β cell function as evidenced by improved glucose tolerance and a trend in increased insulin secretion. When apoptosis was measured in the midst of overt autoimmunity at 7 weeks of age, we found no significant differences between the control and treatment groups, leading us to conclude that the effects of carbamazepine to preserve β cell mass are mild compared to its ability to promote β cell function. Whether the effects of carbamazepine on β cell function are direct or indirect requires further study. Given the limited role for plasma membrane sodium channels in β-cell function and previous work on mouse β cells from our group and others that failed to find stimulatory effects of acute carbamazepine treatment or Nav1.7 gene deletion on insulin secretion (79; 169; 174; 177), we propose that this drug improves β cell health by an indirect molecular mechanism, perhaps by modulating Na⁺ conductance across intracellular membranes (or independent of Nav1.7).

Carbamazepine may also act on other cells of the endocrine pancreas to induce metabolic
effects prior to the onset of diabetes. α cells are the primary secretors of glucagon, where it acts to promote catabolism and increase blood glucose. Notably, hyperglucagonemia is associated with the loss of insulin in type 1 diabetes (199). Mice deficient in the glucagon receptor are protected from hyperglycemia, even after the ablation of pancreatic β cells by streptozotocin, a β cell specific cytotoxic compound (200). Somatostatin, secreted by the delta cell, also suppresses the secretion of glucagon and subsequent hyperglycemia in alloxan (β cell cytotoxic compound) treated dogs (201). Finally, treatment of streptozotocin treated mice with ranolazine, another sodium channel blocker, reduced the secretion of glucagon and hyperglycemia (202). Although our previous in vitro experiments have suggested a role for carbamazepine at the level of the β cell, there is considerable evidence to suggest that the action of carbamazepine on other endocrine cells may explain improvements in glucose homeostasis.

4.2. Proposed Mechanisms of Carbamazepine and its Clinical Relevance

Carbamazepine is clinically used as an anti-epileptic and is believed to act via the use-dependent inhibition of voltage-gated sodium channels (203). Carbamazepine also has additional effects that may or may not be related to its targeting of plasma membrane sodium channels. For example, carbamazepine has been shown to modulate K<sub>ATP</sub> channels (204; 205), GABA<sub>A</sub> receptors (206), and L-type Ca<sup>2+</sup> channels (207). Specifically, carbamazepine has been reported to relieve K<sub>ATP</sub> channel trafficking defects caused by mutating sulfonylurea receptor 1 (SUR1)(204; 208). Carbamazepine can also inhibit K<sub>ATP</sub> channels by reducing the stimulatory abilities of MgADP, reminiscent of sulfonylurea activity (205). There is also literature to suggest the involvement of GABA signalling in pancreatic β cell function, survival and identity (209-211). While we have shown previously that carbamazepine acts on Nav1.7/Scn9a in pancreatic β cells (189), we cannot
rule out additional effects on other ion channels that are relevant to β cell function and health without conducting additional experiments in future studies.

There are also reports of carbamazepine effects that may be independent of plasma membrane electrical activity. In macrophages, Nav1.5 has been proposed to be present on the late endosome and contributes to endosomal acidification (182). Carbamazepine also induces autophagy in hepatic and neuronal tissues, as well as INS-1E cells and human islets, which could theoretically contribute to improved cell health by reducing the severity of protein aggregation (212-214). In mouse pancreatic β cells, autophagy has been shown to maintain insulin secretion (215), unfolded protein response (UPR) machinery (216), and cellular architecture (215; 217). Functionally, loss of autophagy induces hyperglycemia (215; 217) and is associated with decreased β cell mass (215) and increased cell death (218). Interestingly, under some conditions, autophagy may also play a role in β cell death (219). Whether β-cell autophagy represents a target of carbamazepine requires additional study.

Several small epidemiological and clinical studies have linked the incidence of epilepsy to type 1 diabetes (220-223), although it is unclear whether these diseases are linked by an underlying molecular mechanism. Topiramate, another sodium channel blocker and anti-epileptic drug, was administered to a 43 year-old Caucasian patient with recently diagnosed ketotic diabetes as an adjunct to existing valproic acid for generalized seizures, where the use of exogenous insulin was rapidly reduced to 3 units/day and even discontinued after 55 months (224). Sodium channel blockers may offer an additional benefit for type 1 diabetic patients, as the Nav1.7 is principally involved in pain sensing (225). Topiramate, as well as other sodium channel blockers, have already been investigated for the treatment of neuropathic pain in type 2 diabetes (226).
4.3. Alternative Mechanisms of Carbamazepine to Protect Against Type 1 Diabetes

Carbamazepine has been documented to have both inhibitory and activating effects on the immune system (227-229). Therefore, it is unclear whether the protective effects of carbamazepine can be attributed to its influence on the immune system. The main difference in immune activity we observed in our study was decreased insulitis in carbamazepine treated mice as compared to controls. However, many factors are involved in the immune infiltration of islets, and infiltration is not always correlated to destruction of the pancreatic β cells. Insulitis is defined as the infiltration of the islets by mononuclear cells that are largely comprised of T cells, although the presence of other cell types (e.g. B cells and macrophages) have also been reported (230). In high glucose environments, human β cells had increased preproinsulin antigen presentation which coincided with an increase in T cell cytotoxicity (231). Thus, methods to alter the amount of antigen presented to autoimmune cells may prove to be effective in mitigating type 1 diabetes.

We specifically examined several aspects of immune cell activity in the pancreata of carbamazepine-treated NOD mice and their un-treated controls. No overt differences were observed in the proliferation of immune cells in the vicinity of pancreatic β cell cells from qualitative comparisons between histological samples, and changes to the proportions of CD4+ to CD8+ T cells in the draining lymph node were not observed. Unfortunately, attempts to measure degranulation activity in the isolated lymphocytes after activation by PMA and ionomycin were largely unsuccessful. Thus, we were unable to measure direct cytotoxic activity of the main inducers of β cell apoptosis. In addition, because pancreata were used for histological experiments, we opted to measure circulating markers of inflammation and largely found no differences in inflammatory cytokines except for IL-1β, but this difference disappeared later on in the study. Thus, from the studies we conducted, we did not observe robust differences in the activation state...
of the immune system between treatment groups. However, this does not preclude the possibility that carbamazepine affects islet-specific immune cell activity. For example, experiments analysing islet autoantigen specific T cells via tetramer staining, could provide insight into the relative contribution of carbamazepine on islet specific cytotoxic T cell activity. However, such an experiment was outside the scope of this study.

4.4. Limitations of the Study

The primary objective of the study was to examine whether carbamazepine conferred any anti-diabetic effects *in vivo* against a fully competent immune system. Although attempts were made to elucidate the exact protective mechanisms of carbamazepine, our principal mechanistic finding is early evidence of improved glucose tolerance. The molecular mechanisms that confer protection against type 1 diabetes by carbamazepine remain unclear, and will be the subject of investigation in future studies. It is also unknown whether carbamazepine simply delays the incidence of diabetes, or whether it confers lasting protection against its development. Unfortunately, the low sample size relative to what is needed (i.e. to identify differences in certain parameters like glucose stimulated insulin secretion and age-dependent changes in β cell mass) limits the conclusions of this study.

While the majority of our current and previous data point to type 1 diabetes relevant effects of carbamazepine at the level of the pancreatic β cells, we cannot formally exclude the possibility of relevant effects on other cell types, particularly the pancreatic α and δ cells, as well as the immune system. Many facets of both the endocrine pancreas and the immune system have not yet been examined in our model, including the possible action of carbamazepine on: excitability and
secretion of hormonal and paracrine factors, the function of cytotoxic and cytokine-producing T cells, antigen presentation, and the activity of regulatory T cells.

4.5. Future Directions

Our results suggest anti-diabetic effects of carbamazepine that are robust in both in vitro and in vivo models of type 1 diabetes. Carbamazepine is used in the treatment of epilepsy (232), but it is also associated with numerous side effects (233; 234). There is an unmet need for identification and development of carbamazepine-like analogues as a potential new class of novel anti-diabetic drugs, especially in the realm of direct β cell protection. Future work should be undertaken to optimize carbamazepine centric therapy focusing on the elucidation of carbamazepine’s specific target(s) of action as well as the generation of carbamazepine derivatives that are impermeant blood-brain barrier to reduce unwanted central side effects.

In the NOD animal model, animals should be treated at various stages of disease progression (i.e. onset of autoantibody detection versus after the development of chronic hyperglycemia) to determine whether carbamazepine can both prevent and reverse disease at stages that are more clinically relevant to human patients. Larger cohorts of NOD mice will need to be assessed for glucose tolerance and GSIS at various stages of disease progression to allow the tracking of the progression of diabetes in greater detail. These mice should also be euthanized at multiple time points, with the tracking of β cell loss and function extending beyond 25 weeks of age. The nature of any β cell-specific effects by carbamazepine can be assessed with transcriptome sequencing, and promising genetic pathways can be validated with the use of chemical inhibitors and/or knockdown of key players with siRNA. Generic pathways that influence β cell function
and viability, such as ER stress, the unfolded protein response, and autophagy, can be assessed by histochemical methods or by Western blot.

Our working model is that a likely mechanism by which carbamazepine acts to mediate its anti-diabetic effect is via the use-dependent block of voltage gated sodium channels, in particular the Nav1.7 channel, either on the plasma membrane or on the membrane of a relevant organelle such as the autophagosome. In collaboration with Dr. Francis Lynn, we have initiated preliminary experiments to examine MIN6 cells made Nav1.7 deficient with CRISPR. Experiments should initially focus on the viability and function of these MIN6 cells when challenged against inflammatory cytokines in tandem with carbamazepine treatment. Eventually, mice with tissue specific knockout of Nav1.7 backcrossed onto the NOD mouse line with or without treatment of carbamazepine should also be used to determine whether the drug acts principally via the Nav1.7 sodium channel and the contribution of off-target effects to its function.

It will still be important to fully evaluate the role of the immune system in the context of carbamazepine treatment and its ability to inhibit the development of type 1 diabetes. Experiments should be conducted to assess islet specific cytotoxic T cells and T helper cells by tetramer staining to see whether exocytotic activity and production of cytotoxic (e.g. perforin, granzyme, FasL) and inflammatory factors are changed. Further experiments should also look to assess the ability of antigen presenting cells to present cognate antigen and activate accessory cells under the influence of carbamazepine. Migratory behaviour of immune cells with administration carbamazepine can also be assessed to determine whether the protective effects of carbamazepine are in part, due to influences on the immune system. Finally, experiments involving adoptive transfer of purified immune cell populations from carbamazepine treated or untreated NOD mice to recipient NOD-
SCID mice can be used to determine whether the immune system is a major contributor to the protective effects of carbamazepine in type 1 diabetes.

Collectively, the completed and planned work has the potential to establish Na\(^+\) inhibition as a new β cell centric therapeutic modality in type 1 diabetes. If successful, this work may establish a new approach in preserving β cell function and/or survivability in type 1 diabetes.
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