GENE THERAPY FOR DIABETES:
TARGETING THE GUT FOR INSULIN REPLACEMENT

By

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ABSTRACT

Type 1 diabetes mellitus (T1DM), a chronic metabolic disorder characterized by hyperglycemia, is caused by the autoimmune destruction of insulin-producing pancreatic β-cells. The most common current treatment for T1DM is insulin administration; however, ideal blood glucose levels are rarely achieved in these individuals who are therefore at increased risk for developing debilitating complications of diabetes. Gene therapy may provide a means for achieving prolonged regulated production and delivery of insulin within the body. Targeting meal-responsive gut K-cells as an alternative insulin source has been previously demonstrated to protect mice against streptozotocin- (STZ)-induced diabetes. However, since insulin is a potential auto-antigen, genetically modified insulin-producing K-cells may trigger an autoimmune response. In this thesis, the possibility of autoimmune destruction of modified K-cells was investigated using the non-obese diabetic (NOD) mouse model of T1DM. Transgenic NOD mice containing a rat GIP promoter/mouse insulin transgene showed insulin-immunoreactivity in their gut K-cells. However, quantification of these cells indicated that only a portion apparently possessed the proinsulin processing enzymes CPE (39.3%) and/or PC2 (27.8%), suggesting proinsulin processing may be impaired in the majority of K-cells. Regardless, the presence of bioactive insulin coming from the gut of female transgenic NOD mice was evident as these mice maintained lower random blood glucose levels following STZ-treatment (16 ± 6 mM) and had a 2.5-fold lower incidence of spontaneous diabetes (25.5% at 12-months of age) compared to non-transgenic female NOD mice (30 mM and 66.2% at 12-months of age, respectively). Moreover, despite clear autoimmune destruction of β-cells, insulin-producing gut K-cells were still abundant in transgenic NOD mice. Furthermore, when duodenal sections from non-transgenic and transgenic female NOD mice were examined, no detectable differences were observed between the genotypes for villus and crypt lengths and ratio, K-cell distribution along the crypt-villus axis, nor the density of leukocytes along the villus or within the gut epithelium.
Overall, these findings suggest that there is no autoimmune targeting or destruction of insulin-producing K-cells in transgenic NOD mice and thus further support targeting K-cells for insulin replacement as a potential treatment for T1DM.
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<td>Antigen presenting cell</td>
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<tr>
<td>CPE</td>
<td>Carboxypeptidase E</td>
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<td>CTS</td>
<td>Cataract Shionogi</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>GAD</td>
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<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
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<td>GK</td>
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<td>H&amp;E</td>
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<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>INF</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<td>ISH</td>
<td>In situ hybridization</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NOD</td>
<td>Non-obese diabetic</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PC1/3</td>
<td>Prohormone convertase 1/3</td>
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<td>PC2</td>
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<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
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<td>RT-PCR</td>
<td>Reverse-transcriptase polymerase chain reaction</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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<td>Abbreviation</td>
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<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>VNTR</td>
<td>Variable number tandem repeats</td>
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It is hard to believe that it has been two years since I embarked on this journey. Reflecting back, it has definitely been an exciting and rewarding experience. However, the success of this journey would not have been possible without the help and support of numerous people in my work and life. First and foremost, I would like to thank my supervisor, Dr. Timothy Kieffer, for all his guidance and encouragement. Throughout these last three years, you have challenged me to think critically and quickly – pushing me beyond my self-perceived abilities. Your input and honest feedback has been invaluable and are greatly appreciated. Thanks also for your understanding and patience, especially during these last two months as I struggled to complete my thesis. But most importantly, thanks for taking me on as a graduate student and giving me the opportunity to enter and explore the world of research despite knowing that I ultimately hoped to get into medicine.

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INTRODUCTION

Type 1 Diabetes Mellitus

Diabetes mellitus is currently the most common chronic disorder in the world (1) that has become a growing global epidemic as developing countries undergo urbanization and adopt more Westernized lifestyles and as the current population ages (2-5). In fact, the global prevalence and the total number of people with diabetes are projected to increase from 2.8% and 171 million in 2000 to 4.4% and 366 million in 2030, respectively (2). According to the World Health Organization, an estimated 2 million Canadians had diabetes in 2000 and this number is expected to increase to 3.5 million in 2030 (3). The chronic nature and epidemic rise in diabetes is placing a growing burden on the world health care system for health care needs, resource utilization and costs for prevention and treatment of this disease. In Canada alone, treating diabetes and its complications costs the health care system 13.2 billion dollars annually, a figure that is expected to increase to 19.2 billion per annum by the year 2020 (6). Clearly, diabetes has become a major global health concern, stimulating research directed at better understanding the disease and finding potential treatments and cures.

Diabetes is a heterogeneous group of metabolic diseases characterized mainly by elevated blood glucose levels resulting from defects in the secretion and/or action of insulin, a blood glucose-lowering hormone produced by the β-cells of pancreatic islets of Langerhans (7, 8). There are two main types of diabetes, classified based on etiology. Type 2 diabetes mellitus (T2DM) is the more common form of diabetes, accounting for 90% of diabetes cases in humans, and is characterized by resistance to insulin-mediated glucose transport at peripheral tissues including skeletal muscles and adipose tissue; decreased insulin mediated inhibition of hepatic glucose production; and abnormal regulation of insulin secretion (e.g. less sensitive to glucose) (1). In contrast, type 1 diabetes mellitus (T1DM), which accounts for 5-10% of diagnosed cases of diabetes, typically results from autoimmune destruction of the insulin-producing β-cells (9)
following insulitis, which is the process of intra-islet infiltration by mononuclear cells (10). Although most focus has been on the increase in T2DM, there has been a parallel increase in the incidence of T1DM, especially in children < 5 years old (11). Furthermore, the incidence of T1DM varies internationally; for example, children in Finland have almost 40 times greater chance of developing this disease than children in Japan and almost 100 times greater chance compared to children from certain regions of China (11).

Lacking sufficient endogenous insulin production, patients with T1DM rely on carefully regulated daily insulin injections, food intake and physical activity as well as close blood glucose monitoring to control their blood glucose (9). Since ideal blood glucose levels are rarely achieved in these individuals, they are at increased risk for developing serious long-term complications (12). Chronic hyperglycemia leads to long-term damage, dysfunction and failure of various organs. As a result, individuals with diabetes are more susceptible to heart diseases, strokes, end stage renal diseases, blindness and lower limb amputations (1, 8), complications that are largely responsible for the shortened life expectancy of these individuals (1). Therefore, the goal of diabetes therapy is to maintain blood glucose levels as close to normal as possible.

**Current Treatments for Type 1 Diabetes**

Currently, the main treatment for T1DM includes frequent blood glucose monitoring, taking insulin, maintaining a healthy weight, eating healthy and exercising regularly. Since the digestive system produces enzymes that degrade oral insulin, insulin is typically administered by injection with a fine needle and syringe or an insulin pen (13). An alternative to insulin injection is the insulin pump, which is a device that is worn on the outside of the body and delivers insulin through a needle implanted under the skin. The pump is programmed to deliver a predetermined amount of insulin and can be adjusted based on meals, activity levels and blood glucose levels (13). There are many injectable insulin analogues, including rapid-, intermediate- and long-acting forms of insulin, which when used in combinations can provide a
similar level of control and convenience as insulin pumps (11, 13). In addition, inhaled insulin (e.g. Exubera) is now another option that can replace short-acting forms of injectable insulin (14-17). This powdered form of insulin is administered using a hand-held inhaler and is quickly absorbed by the lungs. However, since inhaled insulin is rapid-acting, the use of injectable longer-acting insulin is still required (15-17).

Regardless of the route or type of insulin administered, tight glucose control has been demonstrated to be critical to delaying the onset and reducing the progression of chronic complications of diabetes (12). Compared to patients treated with conventional insulin therapy (which involved 1 to 2 daily insulin injections and daily urine or blood glucose self-monitoring), patients treated with intensive insulin therapy (which included at least 3 daily insulin injections or use of an external pump, the doses of which were adjusted based on the four daily blood glucose values measured) showed delayed onset and slower progression of retinopathy, nephropathy and neuropathy by 35 to over 70% (12). The importance of close coordination of insulin delivery with meals is emphasized by the observation of impaired glucose tolerance and development of diabetic complications resulting from impaired meal-induced insulin secretion (18). In fact, Bastry et al. (19) demonstrated that insulin therapy targeting post-prandial glucose levels resulted in greater reduction of glycosylated hemoglobin (HbA1c) levels and improvement of overall metabolic control than therapy targeting fasting blood glucose.

Although intensive insulin therapy improves glycemic control and reduces long-term complications, it is also associated with extensive body weight gain and increased risks of life-threatening hypoglycemia resulting from insulin over-dosing (12). In light of the limitations of insulin therapy (by needle, pump or inhaler), an attractive approach for treating diabetes is restoring endogenous insulin production. Pancreas (20) and islet transplantations (21, 22) are effective at restoring blood glucose regulation in patients with T1DM and eliminating the need for exogenous insulin. However major drawbacks of both forms of transplantation include the relative lack of organ donors and the need for chronic immunosuppression to prevent rejection.
and recurrent autoimmune islet destruction (13). Several islet transplantation recipients also required multiple transplants, with some transplants requiring more than 1 donor pancreas (22). Furthermore, despite ongoing immunosuppressive therapy, only 10% of patients who had received islet transplants at the University of Alberta maintained insulin independence 5-years post-transplant (22). The short duration of insulin independence and the scarcity of donors have resulted in the search for alternative approaches to treating diabetes.

**Gene Therapy and Diabetes**

The ultimate goal of diabetes therapy is to maintain steady and normal blood glucose levels (fasting blood glucose level < 5.6 mM and 2 hour post-oral glucose load blood glucose < 7.8 mM (7)) by tightly coordinating rapid insulin release with blood glucose-sensing mechanisms. Unfortunately, the main therapy available today for treating T1DM, namely insulin therapy, is unable to meet this goal. With the rapid advances in molecular genetics, gene therapy now offers a promising technology that may address the problems with current diabetes treatments.

Gene therapy involves introducing therapeutic gene(s) into specific cells to correct phenotypic or genotypic abnormalities or to provide cells with new functions (23). The ability to achieve endogenous release of therapeutic proteins from genetically engineered cells within the body makes gene therapy an attractive approach. Thus one strategy to treat diabetes is to target insulin production and secretion to non-islet cells. Such genetically engineered cells are expected to provide a steady insulin supply and thus negate the need for exogenous insulin, while likely avoiding graft rejection and recurring autoimmunity.

Tissues that have been targeted for insulin production include: liver (24), muscle (25), pituitary (26), hematopoietic stem cells (27), fibroblasts (28) and gastrointestinal exocrine glands (29). Together, these studies demonstrate the therapeutic potential of targeting insulin production to non-islet cells, as evidenced by the observed reversal of hyperglycemia and prevention of lethal diabetic complications. However, despite these promising findings, a major
clinical limitation of the above approaches is the lack of glucose-dependency for insulin secretion. To address this concern, some researchers have attempted to regulate insulin gene expression using glucose-sensitive promoters (30). However, the time course of such transcriptional control by glucose is too slow for tight coordination of insulin production with blood glucose fluctuations. Timing of insulin delivery is crucial for optimal glucose homeostasis and tolerance and to avoid episodes of hyperglycemia and hypoglycemia. Therefore, the target cells for ectopic insulin production should ideally be cells that are capable of producing, storing and releasing insulin in a glucose-sensitive fashion.

K-cells as a Target for Gene Therapy in Diabetes

Besides β-cells, there are few glucose-responsive endocrine cells in the body. K-cells, a type of gut endocrine cell found mainly in the epithelial lining of the stomach and upper small intestine (predominantly duodenum and jejunum), secrete the hormone glucose-dependent insulinotropic polypeptide (GIP) in a glucose-responsive manner (31). GIP, a 42 amino-acid peptide, was first isolated from porcine small intestine and was originally referred to as “gastric inhibitory peptide” due to its inhibitory effect on acid secretion in dogs (32). However, studies have since questioned its physiological effects on gastric secretion (33, 34). Instead, due to its ability to enhance insulin release by pancreatic β-cells at physiological doses in the presence of elevated glucose (35-38), GIP is now often referred to as an incretin hormone (38, 39). GIP and glucagon-like peptide-1 (GLP-1), another incretin hormone that is produced by L-cells located in the distal jejunum, ileum, colon and rectum, account for up to 50-60% of the insulin secretory response following oral glucose ingestion and are responsible for the “incretin effect”, which is the observation that compared to intravenously administered glucose of the same concentration, oral glucose administration results in a greater rise in plasma insulin (39). The importance of GIP and its insulinotropic effect on glucose metabolism is highlighted by studies with GIP receptor knockout (GIPR−/−) mice. These mice exhibit glucose intolerance following an
oral load of glucose, demonstrating the role of endogenous GIP at maintaining blood glucose levels following glucose ingestion (40).

Similar to insulin, GIP release following a meal is also dependent on the macronutrients of the meal. GIP release has been mainly demonstrated following ingestions of two major nutrients, carbohydrates and fat. After oral glucose, GIP is rapidly released, preceding that of insulin, reaching peak venous blood levels by 15-30 minutes and returning to basal levels by 180 minutes (41) (Fig. 1A). The type of carbohydrate also affects the magnitude of GIP and insulin release in a parallel fashion. For example, a meal consisting of only glucose elicited the largest increase in circulating GIP, followed by brown rice and then barley (42) (Fig. 1B).

Glucokinase (GK) is the rate-limiting glucose metabolism enzyme that is recognized as the “glucose-sensor” in pancreatic β-cells (43). Therefore, the presence of GK expression in gut K-cells (44) (Fig. 1C) suggests that GK may also confer glucose-sensitivity in K-cells. In contrast to the rapid response to glucose, following oral triglyceride ingestion, peripheral blood GIP levels do not reach peak levels until 120-150 minutes and remain elevated compared to basal levels at 180 minutes (45). Finally, GIP response to protein ingestion has been slightly more controversial. While GIP release in humans is observed following administration of amino acid solutions, no GIP release was reported following various protein meals including steak (46) and cod (47). In dogs, a 10-fold increase in serum GIP levels was observed 15 minutes following intragastric infusion of a protein hydrolysate, peptone, while no change in serum insulin levels was observed (48). In rats, although duodenal mucosal GIP mRNA levels did not change significantly, plasma and duodenal mucosal extract GIP levels increased and peaked (approximately 3 and 2-fold increases, respectively) 120 minutes after intragastric infusion of 10% peptone solution (49).

A comparison of the amount of insulin secreted following an oral glucose challenge to that of GIP suggests that K-cells and β-cells secrete a comparable amount of hormone. In humans with normal glucose tolerance, the amount of GIP secreted in the first 20-30 minutes post-
Figure 1. Comparison of insulin and GIP plasma levels and glucose sensing in K-cells. (A) Mean (± SEM) plasma insulin (circles) and GIP (squares) levels following 75 g glucose ingestion (41). (B) Circulating insulin (green) and GIP (red) levels in healthy human subjects consuming a normal diet. Arrows indicate the start time of meals. (Modified from 42). (C) Co-expression of the glucose-sensor, glucokinase (GK; red) and GIP (green) in gut K-cells (44).
oral ingestion, as indicated by the area under the curve of plasma GIP levels versus time after oral glucose, is 20-55% of that of insulin (50, 51). The observation that clinical symptoms of T1DM do not typically develop until 80% of β-cells have been destroyed (52, 53) indicates that as long as pancreatic insulin secretory potential is at least 20% normal, blood glucose regulation and normal glucose tolerance are maintained. Therefore, if K-cells are modified to produce insulin, sufficient amounts of insulin should be secreted from these cells to regulate blood glucose.

Previous studies in Dr. Timothy J. Kieffer's laboratory have shown that these glucose-sensitive gut K-cells are potential surrogate β-cells (44). Genetic introduction of a rat GIP promoter / human preproinsulin-expressing (rGIP/hlNS) transgene allows K-cells to produce and secrete insulin in a glucose-dependent manner both in vitro with a tumor derived K-cell line (GTC-1) and in vivo with transgenic mice (44). In contrast to the hyperglycemia that is usually observed in wild type mice administered the diabetes-inducing β-cell toxin, streptozotocin (STZ), mice expressing the rGIP/hlNS transgene maintained normal blood glucose levels and glucose tolerance upon treatment with STZ (44). Insulin and blood glucose levels following fasting and re-feeding also demonstrated that insulin production and secretion from K-cells were appropriately regulated by nutritional status (44). In addition, Western blot analysis of GTC-1 cells indicated that K-cells express the enzymes prohormone convertase 1/3 (PC1/3) and PC2, which are required for correct processing of proinsulin to mature insulin (44). The observed presence of PC1/3 and PC2 in GTC-1 cells and protection from chemically-induced diabetes in transgenic mice suggest that insulin is properly processed in the genetically modified K-cells.

The Non-obese Diabetic Mouse

The high-dose STZ model of diabetes, while useful, does not mimic autoimmune T1DM. The non-obese diabetic (NOD) mouse was discovered in 1980 by researchers at the Shinogi Company (54). The NOD mouse was derived from the cataract Shionogi (CTS) mouse and was
established by selectively breeding for spontaneous diabetes and reproductive ability (54). The onset of diabetes is abrupt in both sexes with no observed spontaneous remission (54). Diabetic NOD mice show typical symptoms of diabetes including polyuria, polydipsia, hypoglycemia, glucosuria and hypercholesteremia (54). Similar to patients with T1DM, these mice develop diabetes following lymphocyte infiltration and autoimmune destruction of their β-cells (55) (Fig. 2). Starting at 3-4 weeks, dendritic cells and macrophages, and then T cells (both CD4- and CD8-positive T cells) and B cells infiltrate perivascular duct and peri-islet regions of the pancreatic islets, a process known as peri-insulitis (55). Following this stage, T cells mediate a slow, progressive and selective destruction of insulin-producing β-cells, leading to T1DM onset by 4-6 months of age (55). While both male and female NOD mice develop nondestructive peri-insulitis, insulitis is more invasive and destructive in female NOD mice, resulting in a higher incidence of overt diabetes in female (80-90%) than male (10-40%) NOD mice (55). Both apoptosis and necrosis are believed to be involved in β-cell destruction in response to insulitis (56).

Similar to humans, T cell responsiveness to glutamic acid decarboxylase (GAD) 65 (57, 58) and insulin (59) is detectable in NOD mice and inducing neonatal tolerance to GAD65 prevents development of insulitis and T1DM in these mice (57, 58, 60). However, active immunization with insulin, proinsulin or GAD 65 on their own do not trigger T1DM in NOD mice (61), supporting the hypothesis that T1DM in NOD mice is associated with T cell responsiveness to many antigens (62). Regardless of which autoantigen is/are essential to disease onset, evidence to date suggests that T cell immune dysregulation plays a major role in the initiation of T1DM in NOD mice (55). By 4-6 weeks of age, T cell abnormalities that are observed in NOD mice include T cell proliferative hyporesponsiveness to T cell receptor (TCR) stimulation (63)), reduced IL-2 and IL-4 secretion following T cell activation (64), loss of regulatory or suppressor T cell function and a skewing towards T_{H}1 cells (65). Furthermore, similar to the increased susceptibility to T1DM associated with particular major histocompatibility complex (MHC) class
Figure 2. Immune attack of islets in the pancreas of an NOD mouse. (A) Pancreatic islets under various stages of autoimmune attack. The pancreatic tissue section was stained for insulin (INS; green), glucagon (GLUC; red) and nuclei (DAPI; blue). The arrows indicate islets. (B) A pancreatic islet of an NOD mouse surrounded by immune cells. The pancreatic tissue section was stained for insulin (INS; green), CD45 (red) and nuclei (DAPI; blue). Images were taken at a magnification of 100x (A) and 400x (B).
II alleles in humans, NOD mice possess a MHC haplotype (I-A<sup>q</sup>) that contributes to several dysfunctions of antigen-presenting cells (APC), promoting the development of β-cell-autoreactive T cells (66). Most notably, Carrasco-Marin et al. (67) found that I-A<sup>q</sup> MHC-II molecules bind islet autoantigen peptides with low affinity, leading to inefficient presentation by APCs in the thymus and ultimately failure to delete potentially autoreactive T cells.

Overall, due to the similarities between the NOD mouse and patients with T1DM, the NOD mouse is often used to study the pathogenesis of T1DM as well as to investigate potential treatments and therapies (55). Therefore, the NOD mouse was selected as the animal model of T1DM to study the potential of autoimmune targeting of genetically modified gut K-cells.

**Insulin as a Potential Autoantigen**

The mechanism behind loss of tolerance of lymphocytes towards β-cell antigens during T1DM is still not clear. One hypothesis is that there is either a primary islet abnormality or a primary defect in the immune system (68). An alternative hypothesis is that environmental factors play a role in triggering the disease (68). Adoptive T cell transfer studies in NOD mice and cell-specific cytokine expression studies with transgenic mice (69) along with the recurrence of diabetes in hemipancreas transplants between monozygotic twins that are discordant for the disease suggests that T1DM is highly dependent upon primary defects in the immune systems of patients and animal models of T1DM (68). At the same time, the ability of various viruses to either induce acute diabetes in diabetes-prone animals or protect these animals from developing diabetes reflects the complexity of the influence environmental factors have on disease incidence and progression (68).

However, regardless of the mechanism behind the loss of tolerance in T1DM, what is clear is that T cells and autoantibodies target several autoantigens, which are epitopes on self-proteins that are recognized by the immune system (55). Some of the autoantigens that have been identified include islet cell antigens (e.g. pancreatic sialoglycoconjugate and β-cell glucose
transporter), insulin, GAD 65 and 67, tyrosine phosphatase-like IA-2, and heat shock protein 60 (52, 58, 70-74). In fact, the first autoantibodies detected in children who are at-risk for developing T1DM are anti-insulin antibodies, which are also highly predictive for diabetes in siblings of patients with T1DM (75).

In mice, islet-specific T cell lines established from lymphocytes infiltrating islets of prediabetic NOD mice and clones of these lines showed that insulin-specific T cells are a major component of the spontaneous T cell response to islets in NOD mice (76). Adoptive transfer of clones from these islet-specific T cell lines accelerated diabetes in young NOD mice and adoptively transferred diabetes to NOD/SCID mice (59). Furthermore, injecting prediabetic NOD mice with insulin, insulin B-chain (77) or insulin 2 B-chain peptide 9-23 (78, 79) protected these mice from diabetes.

In humans, the variable number tandem repeat (VNTR) region flanking the insulin gene controls both β-cell response to glucose and the expression of proinsulin in the thymus and alleles in this region confer genetic susceptibility to diabetes (80-82). While there is no VNTR region flanking the insulin gene in the mouse, the mouse expresses two proinsulin isoforms encoded by genes located on chromosomes 7 and 19 (83). These two murine insulin isoforms differ by 2 amino acid residues in B chain (insulin 1: B9 proline, B29 lysine vs. insulin 2: B9 serine, B29 methionine), 3 in C peptide and 6 amino acid residues in the leader sequence (84). The two isoforms also share similar primary structures especially in the A-chain sequence (85). The major difference between the two genes is that while both are comparably expressed in islets, proinsulin 2 expression in the thymus is greater than that of proinsulin 1 (86). The differential involvement of the two isoforms in disease incidence and progression is suggested by the observed accelerated incidence and progression of T1DM and increased production of insulin autoantibodies in proinsulin 2-deficient NOD mice (85) and reduced diabetes and insulitis in proinsulin 1-deficient NOD mice (84).
The relative importance of the various autoantigens is still highly debated. However, in 2005, Nakayama and colleagues presented data that suggested proinsulin as a primary autoantigen in the NOD mouse (87). Based on the finding that mutating native tyrosine at position 16 of the B chain (B16) to alanine eliminated the response of insulin B chain 9-23 peptide (B9-23)-reactive CD4-positive T cell clones, Nakayama and colleagues generated mutated preproinsulin-transgenic NOD mice lacking both native insulin genes and found that these mice did not develop insulin autoantibodies, insulitis or autoimmune diabetes (87). Furthermore, adoptive transfer of splenocytes from insulin gene double knockout mice into immunodeficient NOD/SCID recipients resulted in significantly delayed diabetes onset compared to splenocytes from wild-type NOD mice (87). Similarly, Kent and colleagues (88) found that 50% of clonally expanded lymphocytes isolated from the pancreatic draining lymph nodes of patients with T1DM recognized the insulin A1-15 epitope. In contrast, lymphocytes from healthy subjects failed to show recognition of the insulin A chain fragment. Thus these findings suggest that insulin may indeed be an essential autoantigen causing autoimmune diabetes in NOD mice and humans.

Unlike β-cells, K-cells are not targeted by the autoimmune system of patients with T1DM, as suggested by the persistence of unaltered GIP secretion despite lack of insulin in these patients (89). However, in light of the evidence pointing towards insulin and proinsulin as potentially essential autoantigens, genetic modification of K-cells to produced insulin may render these cells susceptible to autoimmune attack (8). Since the gut renews itself every few days (90) autoimmune targeting of K-cells may result in chronic inflammation of the gut, which may limit the therapeutic benefits of targeting insulin production to the K-cells (8). Thus determining whether genetically modified insulin-secreting K-cells are targeted by the autoimmune system in a mouse model of T1DM is an important step in the development of this approach as a potential treatment for diabetes.
**Type 1 Diabetes and the Gut Immune System**

Many studies have provided evidence for the involvement of the gut immune system in the development of T1DM. Firstly, in rodent models of T1DM (e.g. NOD mice and BioBreeding rats), diet influences the development of autoimmune diabetes (91-93). Secondly, T cells infiltrating islets express the gut associated homing receptor, β7 integrin, and blocking this receptor or its endothelial ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), with antibodies prevents the development of spontaneous diabetes in NOD mice (94-96)). Thirdly, autoimmune diabetes is transferred with mesenterial lymphocytes from young NOD mice to recipients, suggesting that diabetogenic T cells are present in the gut-associated immune system (97). Lastly, oral ingestions of autoantigens influences (either accelerates or prevents) diabetes development in animal models of this disease (98, 99).

Similarly, in humans, a link between T1DM and the gut immune system has been suggested. Although prospective studies conducted in the USA (100, 101) and Germany (102) failed to document any effects on disease of early cow’s milk exposure or breast feeding, epidemiological studies conducted in Finland suggested the potential for an association between early exposure to cow milk formulas with increased risk for developing T1DM in genetically susceptible children (103-105). In addition, serologic studies suggest that newly diagnosed T1DM patients have increased immune responses to several cow milk proteins (106-108). GAD-specific T cells of patients with T1DM have also been found to express gut-associated homing receptor α4β7-integrin (109) and T cells derived from pancreas of these patients show gut mucosal homing properties (110).

The relationship between gut immunity and T1DM was further supported by studies with oral insulin in the NOD mouse. When oral insulin was given to NOD mice prior to the development of diabetes, disease onset was delayed (111), (112). Oral administration of insulin is believed to mediate this tolerogenic effect by inducing the generation of regulatory T cells in the spleen where they down-regulate effector T cells by blocking cytokine secretion and the
migration of effector T cells to the pancreas (113). Based on these findings with NOD mice, several multi-center randomized clinical trials were conducted. However, in contrast to the protective effects observed in NOD mice, these clinical trials found that treatment with oral insulin did not slow disease progression, as indicated by \( \beta \)-cell function, in patients with recent-onset T1DM (114, 115) nor did it prevent or delay diabetes in individuals at risk for T1DM (116).

Considering the link between the pancreas and gut immune system and the concept of insulin as an essential autoantigen in T1DM, it seems possible that activated T cells derived from the pancreas may recognize and destroy insulin-producing K-cells. It may also be possible that gut immune cells that normally recognize and destroy pancreatic \( \beta \)-cells will also recognize insulin-producing K-cells, leading to continuous targeting and destruction of the K-cells.
THESIS INVESTIGATION

Approximately 5-10% of people diagnosed with diabetes have T1DM, the incidence of which is increasing annually worldwide. Given that current treatments fail to properly mimic the tight coordination of insulin secretion with blood glucose fluctuations and that islet and pancreas transplantations are associated with donor shortage, the need for life-long immunosuppression and recurrence of insulin dependency, there is a great impetus for alternative therapeutic options. An attractive approach is restoring endogenous insulin production via gene therapy. A promising target for meal-responsive insulin production is the gut K-cell. Studies have shown that transgenic mice expressing modified insulin-producing K-cells are protected from developing STZ-induced diabetes. Furthermore, in vitro studies using a K-cell tumor cell line suggest that these cells have the necessary enzymes to sense glucose and properly convert proinsulin into insulin. However, considering the fact that insulin and its precursor have been shown to be autoantigens and that there is evidence for communication between the gut immune system and the pancreas, a potential drawback of targeting the gut K-cells for insulin replacement is autoimmunity. Therefore the goal of this thesis was to examine the possibility of immune targeting and destruction of modified insulin-producing gut K-cells in a mouse model of T1DM, the NOD mouse.

The overall hypothesis of this research was that genetically modified insulin-secreting K-cells can functionally replace \( \beta \)-cells as a source for endogenous insulin without evoking an autoimmune response in NOD mice. To address this hypothesis, 3 specific aims were examined in this thesis. First, experiments were conducted to investigate insulin protein expression in the gut of transgenic NOD mice. Secondly, glucose homeostasis and incidence of diabetes was assessed in transgenic NOD mice. Lastly, whether insulin-producing K-cells were, like \( \beta \)-cells, subjected to autoimmune attack in transgenic NOD mice was investigated.
MATERIALS AND METHODS

Animals

Transgenic NOD mice were generated at the University of Alberta by standard procedures. Briefly, the rat GIP promoter / mouse preproinsulin II (rGIP/mlNS) transgene depicted in Figure 3A was injected into NOD embryos. From positive founders, 5 lines of transgenic NOD mice were established and non-transgenic littermates were also bred to serve as controls. Insulin production in K-cells was confirmed by the cellular co-localization of immunoreactive GIP and insulin (Fig. 3B and 3C). Following preliminary results and based upon breeding success, Line 1 was selected for the following studies.

Animals were housed (2 to 4 mice per cage) in a 12 hour light/dark cycle and given standard mice chow (Research Diets Inc., New Brunswick, NJ, USA) and water ad libitum. For breeding purposes, female NOD mice (5-6 weeks of age) were obtained from Jackson Laboratories (Bar Harbour, Maine, USA). All animal research protocols were approved by the Animal Care Committee of the University of British Columbia in compliance with the guidelines of the Canadian Council for Animal Care.

Genotyping by PCR

Mice were weaned at 3 weeks of age and genotyped at 4-5 weeks of age. DNA samples were extracted from tail or ear clips using Qiagen’s DNeasy® Tissue Kit (Qiagen, Mississauga, Ontario, Canada) or 2% Chelex® 100 Resin (Bio-Rad Laboratories Ltd., Hercules, CA, USA) solution containing 1% proteinase K and 0.1% Tween 20, respectively. DNA samples (5 µL for tail DNA or 3 µL for ear DNA) were then subjected to polymerase chain reaction (PCR) using the following primers: forward primer RGIPpF1 (-TTCCGGAGTGCCACCATT-), which is located in the GIP promoter (-444 to -424 bp) and reverse primer MINSR1 (-CCTGGAAGATAG-GCTGGGTTG-), which is located in the insulin sequence (+148 to +168 bp), amplifying a product with a predicted size of 612 bp. The PCR protocol used is as follow: heat to 95°C for 5
Figure 3. Targeting insulin production to K-cells. (A) Rat GIP promoter / mouse preproinsulin (rGIP/mlNS) transgene. Exons 1-3 (E1-3) are as indicated. Modified from 26 and 44. (B) Gut villi from a transgenic NOD mouse stained for insulin (INS; green; left image) and GIP (red; middle image). The image on the right is an overlay of the insulin and GIP images, where orange cells (indicated by arrows) are K-cells co-expressing insulin and GIP. Images were taken at a magnification of 200x. (C) On the left is an image of a gut villus stained for insulin (INS; green), GIP (red) and nuclei (DAPI; blue) (Magnification = 400x); enlarged images of the boxed area shows a K-cell co-expressing insulin (INS; green) and GIP (red), as indicated by the orange staining observed in the overlay image on the right.
minutes; 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds and then 72°C for 1 minute; and finally 72°C for 10 minutes and was performed with a Peltier model PTC-200 thermal cycler (MJ Research, Inc., Waltham, MA USA). The samples were then separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and imaged with QuantityOne Software (Bio-Rad Laboratories Ltd.).

Characterization of Gut Insulin Protein Expression – Preliminary Studies

Tissue harvest

Animals (3 female transgenic NOD mice, aged 6-7 months) were anesthetized with isoflurane and the pancreas and entire small intestine were harvested and fixed as described above. Following washes with 70% ethanol, the small intestine was rinsed with 1x PBS using an 18G-needle attached to a 5 ml syringe to remove most food traces. The gut was then cut into segments of 2-2.5 cm (the proximal of each segment was indicated with a piece of knotted thread) and stored in Eppendorf tubes containing 70% ethanol until taken over to the UBC Department of Pathology and Laboratory Medicine for processing and sectioning as described above.

Immunohistochemistry

Tissues sections were prepared and stained as detailed in the “General Immunohistochemistry Procedure” section below. Using pancreas sections as a positive control, the gut segments were stained with the following primary antibodies: guinea pig anti-insulin (1:900; Linco); mouse anti-insulin (MAb1; 1:100; Biodesign International, Saco, ME, USA); rabbit anti-carboxypeptidase E (CPE, also known as CPH; 1:100; AbD Serotec, Raleigh, NC, USA); and rabbit anti-PC2 (1:300; ABR-Affinity Bioreagents Inc., Golden, CO, USA). Gut segments were also co-stained with guinea pig anti-insulin (1:900; Linco) and rabbit anti-GLP-1(1-37)-Amide (1:250; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). Details about these primary antibodies including specific staining procedures are summarized in Table 1. The secondary
antibodies used were: Alexa Fluor 488 goat anti-guinea pig (Linco insulin); Alexa Fluor 594 goat anti-mouse (MAb1); and Alexa Fluor 594 donkey anti-rabbit (CPE, PC2 and GLP-1).

The distributions of gut cells immunoreactive for insulin, GLP-1 and both insulin and GLP-1 were determined by visually scanning each gut tissue sections at a magnification of 400x from the proximal to distal ends of each section. Altering between the green and red filters, the numbers of cells that were positive for insulin, GLP-1 or both insulin and GLP-1 were visually counted. For each gut segment, these counts were then presented as a percent of all cells counted (i.e. number of cells that were insulin- and/or GLP-1- positive).

Table I. The specificity, supplier and immunostaining procedure details of the primary antibodies used.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>SUPPLIER</th>
<th>SOURCE</th>
<th>SPECIFICITY</th>
<th>ANTIGEN RETRIEVAL</th>
<th>DILUTION</th>
<th>INCUBATION DURATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AnaSpec Incorporated</td>
<td>Rabbit</td>
<td>T cells</td>
<td>3 x 5 minutes</td>
<td>1:100</td>
<td>Overnight</td>
</tr>
<tr>
<td>CD45 (LCA) Ly-5 Clone: 30 F-11</td>
<td>BD Biosciences Pharmingen</td>
<td>Rat</td>
<td>Leukocytes</td>
<td>4 x 5 minutes</td>
<td>1:25</td>
<td>Overnight</td>
</tr>
<tr>
<td>CPE (CPH)</td>
<td>AbD Serotec, Inc.</td>
<td>Rabbit</td>
<td>Mature N-Terminal of CPE</td>
<td>3 x 5 minutes</td>
<td>1:100</td>
<td>Overnight</td>
</tr>
<tr>
<td>GIP 3.65 H</td>
<td>Dr. Buchan, University of B.C., Vancouver, Canada</td>
<td>Mouse</td>
<td>C-terminus of GIP (1-42)</td>
<td>3 x 5 minutes</td>
<td>1:1000</td>
<td>Overnight</td>
</tr>
<tr>
<td>GIP</td>
<td>Yanaihara Institute Inc.</td>
<td>Rabbit</td>
<td>Central portion of GIP</td>
<td>3 x 5 minutes</td>
<td>1:1000</td>
<td>Overnight</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Phoenix Pharmaceuticals, Inc.</td>
<td>Rabbit</td>
<td>GLP-1(7-36)-Amide</td>
<td>3 x 5 minutes</td>
<td>1:250</td>
<td>Overnight</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Sigma</td>
<td>Mouse</td>
<td>-</td>
<td>3 x 5 minutes</td>
<td>1:900</td>
<td>Overnight</td>
</tr>
<tr>
<td>Insulin</td>
<td>Linco</td>
<td>Guinea Pig</td>
<td>-</td>
<td>3 x 5 minutes</td>
<td>1:900</td>
<td>Overnight</td>
</tr>
<tr>
<td>Insulin (MAb1).</td>
<td>Biodesign International</td>
<td>Mouse</td>
<td>B Chain C-terminus [split-65,66 PI, des-64,65 PI and mature insulin]</td>
<td>3 x 5 minutes</td>
<td>1:100</td>
<td>3 nights</td>
</tr>
<tr>
<td>PC 2</td>
<td>ABR-Affinity Bioreagents Inc.</td>
<td>Rabbit</td>
<td>PC2 amino acid residues 622-638</td>
<td>3 x 5 minutes</td>
<td>1:300</td>
<td>Overnight</td>
</tr>
</tbody>
</table>
To quantify the percentage of Linco insulin-positive cells that were also positive for MAb1 and the proinsulin processing enzymes CPE and PC2 (see Figure 4 for binding specificity of MAb1, as indicated by the manufacturer’s product data sheet, and the location of these enzymes in the proinsulin processing pathway (117)), the gut tissue sections were scanned as described above. The number of Linco insulin-positive cells (green) and the number of these cells that were also positive for MAb1, CPE or PC2 (red) were visually counted. For each gut segment, the percent of Linco insulin-positive cells co-positive for MAb1, CPE or PC2 was then calculated.

For each of the above quantifications, one section of each gut segment was stained and quantified for each mouse. Observed fluorescence was considered as a positive event (cell) if: 1) the observed fluorescence was confidently above background fluorescence of the tissue; 2) the fluorescence corresponded to a cell (i.e. located around/near a DAPI-positive nucleus); and 3) the fluorescence pattern observed was unique to the specific filter.

**Streptozotocin-Induced Diabetes – Preliminary Studies**

**Streptozotocin (STZ) treatment**

Three non-transgenic (2 females and 1 male) and 7 transgenic (3 females, 4 males) 2.5-month old NOD mice were treated with a single dose of STZ (180 mg/kg body weight) by intraperitoneal (IP) injection. Random blood glucose was monitored daily for the first week following STZ treatment and then twice a week for 2 weeks thereafter. Blood glucose was measured from the tail vein using a portable OneTouch® Ultra® glucometer (LifeScan Inc., Milpitas, CA, USA).

**Tissue harvest and terminal blood sample**

At the end of the study, animals were anesthetized with isoflurane (Baxter Corporation, Mississauga, ON, Canada). Pancreas and duodenal sections were harvested, rinsed with 1x phosphate buffered saline (PBS) and fixed overnight in 4% buffered paraformaldehyde at 4°C.
Figure 4. Schematic diagram of proinsulin processing. As shown, the conversion of proinsulin to insulin occurs by a branched pathway where the prohormone convertases PC1/3 and PC2 cleave proinsulin between Arg\(^{32}\)Arg\(^{33}\) and Arg\(^{65}\)Gly\(^{66}\), respectively. Following cleavage by the PC enzymes, the exopeptidase, carboxypeptidase E (CPE, also known as CPH) removes the exposed dibasic residues. An antibody specific to the processed B-chain / C-peptide junction (MAb1) is indicated in green on the diagram; the cross-reactivity of this antibody with the various intermediates is indicated in blue. Modified from 117.
The following day, the samples were washed 3 times with 70% ethanol and then stored in 70% ethanol until taken over to the UBC Department of Pathology and Laboratory Medicine (Vancouver, British Columbia, Canada) where the samples were paraffin-embedded and sectioned into 3 μm-thick sections (2 sections per slide, either two pancreas, two duodenal or one pancreas and one duodenal sections per slide). Slides were stored at room temperature until immunohistochemistry could be performed on the sections to determine insulin and glucagon (pancreas) or GIP (duodenum) protein presence and localization.

**Immunohistochemistry of pancreas and duodenal sections**

The tissue sections were prepared as outlined in "General Immunohistochemistry Procedure" below. The primary antibodies used were guinea pig anti-insulin (1:900; Linco Research, St Charles, MO, USA), mouse anti-glucagon (1:900; Sigma, St. Louis, MO, USA) and mouse anti-GIP (1:1000; graciously provided by Dr. A. Buchan, University of British Columbia, Vancouver, Canada (118)). The secondary antibodies used were Alexa Fluor goat anti-guinea pig 488 and Alexa Fluor goat anti-mouse 594 (Invitrogen Life Technologies, Carlsbad, CA, USA). Specific immunostaining protocol details pertaining to these primary antibodies are summarized in Table 1.

**Diabetes Incidence**

The non-breeding NOD mouse colony was monitored on a monthly basis in the morning for up to 12 months of age. Random-fed blood glucose was measured via the tail vein as described above. To determine the incidence of diabetes in the mice, survival curves for male and female NOD mice were generated using the Prism software package (Version 4.0, GraphPad, San Diego, California, USA). Animals that became diabetic were given a value of “1” while non-diabetic mice taken early for other studies were given a value of “0” at the time of removal from diabetes incidence monitoring.
Long-Term Tracking for Autoimmunity

**Tissue harvest**

Female transgenic mice and their non-transgenic littermates were sacrificed at 4, 6, 7, 8, 10 and 12 months of age. Random blood glucose was measured from the tail vein as described above. Mice were anesthetized with isoflurane and the pancreas and duodenum were then harvested, fixed and processed as described above.

**Immunohistochemistry**

For all immunofluorescence staining, tissue sections were prepared as outlined in "General Immunohistochemistry Procedure" below. Primary antibodies used for staining and specific protocol details pertaining to the use of these antibodies are summarized in Table 1.

**Evaluation of insulin content of pancreas and duodenum**

Pancreas and duodenal sections from NOD mice of various ages were stained for insulin (Linco) and glucagon (Sigma; pancreas) or GIP (Buchan; duodenum). The secondary antibodies used were Alexa Fluor goat anti-guinea pig 488 and Alexa Fluor goat anti-mouse 594. The sections (1-3 per mouse) were then examined under a fluorescence microscope for insulin immunoreactivity. Signs of lymphocyte infiltration in the pancreas sections were also noted.

**Quantification of villus and crypt lengths and ratio**

Duodenal and pancreas sections from 10 non-transgenic and 12 transgenic female NOD mice were stained with hematoxylin and eosin (H&E) as described below under “General Immunohistochemistry Procedure”. Images of the sections were taken at 100x magnification and 6-53 villi per duodenal section per mouse (average 26 and 22 villi for non-transgenic and transgenic sections respectively) were analyzed, depending on the quality of the tissue sections. Villus and crypt lengths were measured using the OpenLab modular software for scientific imaging (version 5, Improvision Inc, Lexington, MA, USA) as shown in Figure 5. Briefly, the
Figure 5. Schematic diagram depicting how villus and crypt measures were determined. Duodenal tissue sections, stained with hematoxylin and eosin, were imaged at a magnification of 200x. Villi and crypt lengths were then measured using the OpenLab software. Two measures were taken of each villus (Va and Vb) and crypt (Ca and Cb) and then averaged to obtain a villus and crypt length, respectively.
length of the crypt and villus of each villus was measured on both sides of the villus and averaged. To determine total crypt-villus length the measured crypt and villus lengths were summed for each villus, while villi to crypt ratio was determined by dividing the averaged villus length by the averaged crypt length of each villus. The lengths and ratios of each duodenal section of a mouse was averaged for an n=1. One duodenal section of each mouse was analyzed.

**Location of GIP-positive cells along the crypt-villus axis**

Duodenal sections from 10 non-transgenic and 12 transgenic NOD mice were stained for GIP using a rabbit anti-GIP (1-42) antibody (1:1000; Yanaihara Institute Inc., Shizuoka, Japan). Unlike the N-terminus specificity of the mouse anti-GIP antibody (GIP 3.65 H; Buchan), this antibody recognizes a central region of the GIP molecule and when double-stained with the mouse anti-GIP antibody, reveals a population of GIP-immunoreactive cells that do not stain positive with the mouse anti-GIP antibody (Fig. 6). This observation suggested that this rabbit anti-GIP antibody captures a larger population of GIP-expressing (and thus insulin-expressing) gut cells and was therefore used in this study instead of the mouse anti-GIP antibody. Alexa Fluor 594 donkey anti-rabbit antibody was used as the secondary antibody.

To measure the location of GIP-positive cells along the crypt-villus axis, fluorescence and dark field images were taken at a magnification of 100x, and the distance from the crypt base to GIP-positive cells and to the tip of the villus were measured using the OpenLab software (Fig. 7). The location of the GIP-positive cells was then expressed as percent of the crypt-villus length (i.e. distance of GIP-positive cells from crypt base divided by distance of villus-tip from crypt base). Six to 49 (average of 21 and 29 for non-transgenic and transgenic mice duodenal sections respectively) GIP-positive cells were measured per duodenal section per mouse, again depending on the quality of the tissue sections. The measurements were pooled according to genotype, namely non-transgenic vs. transgenic, and compared in a scatter-plot. The
Figure 6. Two populations of GIP-expressing cells as indicated by co-immunostaining with 2 anti-GIP antibodies with different binding specificities. Duodenal sections from a transgenic mice was double-stained with a GIP antibody specific to the central portion of GIP (Yanaihara; green) and a GIP antibody specific to the C-terminus of GIP (3.65H; red). The yellow arrows indicate cells positive for both GIP antibodies; the white arrow indicates a cell only immunoreactive with the centrally-directed GIP antibody (Yanaihara). Images were taken at a magnification of 400x.
Figure 7. Schematic diagram depicting how K-cell location along the crypt-villus axis was determined. Duodenal sections were stained for GIP (red) and nuclei (DAPI; blue) and were imaged at a magnification of 100x. The distances of GIP-positive cells from the base of the crypt and crypt-villus length were measured using the OpenLab software. To determine the location of a K-cell (indicated by the white arrow in the diagram above), two crypt-villus length measures (CVa and CVb) were taken of the villus and then averaged. The distance of GIP-positive cell from the crypt base (K) was measured and then expressed as a proportion of the averaged crypt-villus length.

Crypt-villus Length = (CVa+CVb)/2
K-cell Location = K / Crypt-villus Length
Proportion of GIP-positive cells located along the bottom (0-33.3%), middle (33.4-66.6%) and top (66.7-100%) portions of the crypt-villus axis was also determined for each section and averaged by genotype. One duodenal section per mouse was analyzed.

**Quantification of general leukocytes and T cells in the duodenum**

Duodenal and pancreas (positive control) sections from 4 transgenic and 4 non-transgenic female NOD mice were stained for general leukocytes and T cells using a rat anti-CD45 (1:25; BD Biosciences Pharmingen, San Jose, CA, USA) and a rabbit anti-CD3 (1:100; AnaSpec Inc., San Jose, CA, USA) antibody, respectively. Pancreas sections were co-stained for insulin using a guinea pig anti-insulin antibody (1:900; Linco) while duodenal sections were co-stained for GIP using a mouse anti-GIP antibody (1:1000; Buchan). Sections were incubated with the following secondary antibodies: Alexa Fluor 594 donkey anti-rat (CD45); Alexa Fluor 594 donkey anti-rabbit (CD3); Alexa Fluor 488 goat anti-guinea pig (insulin); and Alexa Fluor 488 goat anti-mouse (GIP).

For CD45 quantifications, images were taken at a magnification of 200x as described under the “General Immunohistochemistry Procedure” section below. CD45-positive cells were then counted and normalized to villus length, measured using the OpenLab software. For each section, 8 to 10 villi were imaged and quantified and one section from each mouse was analyzed.

For CD3 quantifications, images were taken at a magnification of 400x. The number of CD3-positive intra-epithelial cells were counted and normalized to 100 epithelial cells. A minimum of 1000 epithelial cells were counted per section and one section from each mouse was analyzed.
General Immunohistochemistry Procedure

**Fluorescence staining**

The tissue sections were dewaxed and rehydrated in a series of 6-7 minute incubations in xylene and decreasing concentration of ethanol as follows: 3 times xylene, 2 times 100% ethanol, 95% ethanol and 70% ethanol. The sections were then washed with 1x PBS and subjected to heat-induced (microwave, power level 4) antigen retrieval in 10 mM citrate buffer solution, pH 6, for empirically determined optimal times (either three times 5 minute cycles or four times 5 minute cycles). Following antigen retrieval, sections were cooled to room temperature, washed with milli-Q water and 1x PBS for 5 minutes each and then blocked for 30-35 minutes with Serum-Free Protein Block (DakoCytomation, Inc., Carpinteria, California, USA). Sections were then incubated overnight or three nights at 4°C with primary antibody, diluted with Antibody Diluent (DakoCytomation, Inc.). The next day, sections were washed in 1x PBS for three times 5-10 minutes and then incubated with secondary antibody (diluted 1:400 with Antibody Diluent) at room temperature for 1 hour. Finally, sections were washed 3 times in 1x PBS for 5-10 minutes each and then mounted with microscope cover glass using Vectashield® Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). The length of microwaving, antibody dilution and duration of antibody incubation for the primary antibodies used in this thesis are summarized in Table 1.

**Hematoxylin and eosin staining**

Tissue sections were dewaxed and rehydrated as described above. Following the 1x PBS wash, sections were incubated with hematoxylin nuclear counterstain (Gill's Formula; Vector laboratories) for 2.5-3 minutes and then rinsed with running water. Slides were then incubated with eosin for 45 seconds to a minute and then rinsed with running water. Finally, slides were dehydrated in a series of 2 minute incubations in increasing concentrations of ethanol and xylene as follows: 70% ethanol, 95% ethanol, 2 times 100% ethanol, and 3 times xylene. Slides
were then mounted with cover glass using VectaMount Mounting Medium (Vector Laboratories, Inc.) and dried in a 60°C oven for 45 minutes.

**Visualization and image capturing**

Stained tissue sections were visualized using a Carl Zeiss Axiovert 200 inverted research microscope (Carl Zeiss Canada Ltd., Port Moody, BC, Canada) and analyzed using OpenLab modular software. Pictures of the slides were captured using a Retigar 1300R Fast 1394 camera (Qimaging™, Burnaby, BC, Canada).

**Data Analysis**

Unless otherwise stated, data are presented as mean ± standard error of the mean (SEM) with the number of replicates shown in the figure legends. When sample size was < 3, values were averaged and the average or individual values were presented. Data were assessed using Logrank test or Student’s t-test and was considered statistically significant when $P < 0.05$. Data analysis was performed using the Prism software package.
RESULTS

STUDY 1 – Characterization of Gut Insulin Protein Expression (Preliminary Results)

1.1 Distribution of Insulin- and GLP-1-immunoreactive cells

By immunohistochemistry, insulin- and GLP-1-immunoreactive cells were found throughout the small intestine (Fig. 8). To characterize insulin expression in the gut, the distribution of insulin protein expression along the length of the small intestine was compared to that of GLP-1 protein expression. In terms of insulin and GLP-1 expression, 3 populations of cells were observed in the small intestine: cells that only showed insulin or GLP-1-immunoreactivity and cells that showed immunoreactivity towards both proteins (Fig. 8). Quantification of these 3 populations (Fig. 9) revealed that the proportion of solely insulin-immunoreactive cells (i.e. K-cells) was highest in the duodenum (73.5 ± 3.0% total cells counted) and gradually decreased to the proximal ileum (60.9 ± 3.3% total cells counted) and then rapidly decreased towards the distal end of the small intestine (11.9 ± 4.1% total cells counted). Conversely, the proportion of solely GLP-1-immunoreactive cells (i.e. L-cells) gradually increased from the duodenum (5.7 ± 1.1% total cells counted) to the proximal ileum (36.7 ± 2.2% total cells counted) and then increased sharply towards the end of the small intestine (88.1 ± 4.1% total cells counted). The proportion of insulin and GLP-1 double-stained immunoreactive cells increased slightly from the duodenum (20.8 ± 4.0% total cells counted) to the proximal jejunum (27.2 ± 3.5% cells counted) and then gradually decreased to the proximal ileum (12.4 ± 2.0% cells counted) before dropping rapidly to none detected at the end of the small intestine.

1.2 Evaluation of Insulin Processing in Gut K-cells

Given that the structure of insulin affects its biological activity (119, 120), the goal of this study was to examine various aspects of insulin processing in the K-cells of transgenic NOD mice. For this set of experiments, the small intestines of 3 transgenic mice were stained for
Figure 8. Colocalization of insulin- and GLP-1-immunoreactivity in a portion of insulin-immunoreactive gut cells of transgenic NOD mice. Duodenal sections were stained for insulin (INS; green), GLP-1 (red) and nuclei (DAPI; blue). The white arrows indicate cells solely immunoreactive for either insulin or GLP-1; the yellow arrows indicate cells co-expressing insulin and GLP-1. Images were taken at a magnification of 200x (top panel) and 400x (bottom panel).
Figure 9. Distribution of insulin- and/or GLP-1-immunoreactive cells along the small Intestine of transgenic NOD mice. The small intestines of 6-7 month old female transgenic NOD mice were cut into 2-2.5 cm segments, paraffin embedded and sectioned. The gut sections were stained with anti-insulin (Linco) and anti-GLP-1 antibodies. The proportion of immunofluorescent cells that were solely insulin-positive (filled circles), solely GLP-1-positive (filled squares) and insulin- and GLP-1-co-positive (open triangles) was determined and is presented in the graph as mean ± SEM. n = 3.
insulin (Linco insulin antibody) and either insulin processed at the proinsulin B chain / C-peptide (BC) junction (MAb1 antibody), CPE or PC2 (refer to Fig. 4 for diagram of proinsulin processing).

1.2.1 Quantification of insulin processed at the BC junction

Processing at the BC junction was assessed by staining the gut with an antibody that recognizes the various processed forms of insulin (Linco insulin antibody) and an antibody specific to the C-terminus of the B-chain of insulin (MAb1). As shown in the representative images in Figure 10, while β-cells in the pancreas stained strongly for both Linco insulin and MAb1, MAb1 staining in the gut was comparatively much weaker, often difficult to locate without first identifying the Linco insulin-positive cells, and was detectable in only a subset of Linco insulin-positive cells. MAb1 immunoreactivity was also typically more concentrated on the basal side of the cell. From the duodenum to mid-ileum, an average of 20.1 ± 1.8% of Linco insulin-positive cells were positive for MAb1; in the distal ileum, the percent of insulin-positive cells that were also MAb1-positive decreased to 3.2 ± 1.2% (Fig. 11).

1.2.2 Quantification of CPE

Considering the decreased cross-reactivity of the MAb1 antibody to insulin molecules still containing the two arginine residues at the end of the B-chain (refer to Fig. 4), the presence of CPE in insulin-positive cells was assessed. Figure 12 shows representative images of pancreas and duodenal sections stained for Linco insulin and CPE. Unlike the islet, where CPE staining was observed in most, if not all, insulin-positive cells as well as in the surrounding peripheral cells (presumably α-cells), in the gut, only a subset of insulin-positive cells were co-CPE-positive, the proportion and distribution of which varied greatly between mice. In fact, from the duodenum to distal jejunum, only 39.3% of insulin-positive cells were also CPE-positive (Fig. 13). Distal to the jejunum, the percentage of co-CPE-positive insulin-positive cells decreased from 32.5% in the proximal ileum to 4.7% in the last segment of the small intestine (Fig. 13).
Figure 10. MAb1 staining of pancreatic and duodenal tissue section from a 6-month old female transgenic NOD mouse. Tissue sections were stained for insulin (INS; green), insulin processed at the proinsulin B chain / C-peptide junction (MAb1; red) and nuclei (DAPI; blue). The white arrow indicates an insulin-positive cell that was MAb1-negative; the yellow arrows indicate insulin-positive cells that were also MAb1-positive. Images were taken at a magnification of 200x for pancreas and 400x for duodenum.
Figure 11. Quantification of insulin-positive gut cells that were co-MAb1 positive along the small intestine of transgenic NOD mice. The small intestines of 6-7 month old female transgenic NOD mice were cut into 2-2.5 cm segments, paraffin embedded and sectioned. The gut sections were stained for insulin (Linco) and BC junction-processed insulin (MAb1). The percent of insulin-positive cells that were also MAb1-positive was then determined and is presented in the graph as mean ± SEM. n = 3.
Figure 12. CPE staining of pancreatic and duodenal tissue sections from a 6-month old female transgenic NOD mouse. Tissue sections were stained for insulin (INS; green), carboxypeptidase E (CPE; red) and nuclei (DAPI; blue). The white arrow indicates a CPE-positive cell that was insulin-negative; the yellow arrows indicate insulin-positive cells that were also CPE-positive. Images were taken at a magnification of 200x for pancreas and 400x for duodenum.
Figure 13. Quantification of insulin-positive gut cells that were co-CPE positive along the small intestine of transgenic NOD mice. The small intestines of 6-7 month old female transgenic NOD mice were cut into 2-2.5 cm segments, paraffin embedded and sectioned. The gut sections were stained for insulin (Linco) and CPE. The percent of insulin-positive cells that were also CPE-positive was then determined and is presented in the graph for each mouse. n =2.
1.2.3 Quantification of PC2

Since no antibody specific to insulin processed at the proinsulin A chain / C-peptide (AC) junction was located, this study next examined insulin-positive cells for the presence of PC2, the AC junction-processing enzyme. Representative images of pancreas and duodenal sections stained for insulin (Linco) and PC2 are shown in Figure 14. Similar to both MAb1 and CPE staining, while insulin-positive pancreatic islets showed strong staining for PC2, only a portion of insulin-positive cells in the gut showed presence of PC2 immunoreactivity. However, unlike MAb1 and CPE, there appeared to be a slight trend of increased percentage of insulin-positive cells that are also PC2-positive from duodenum (27.8%) to distal ileum (58.9%) (Fig. 15). A comparison of the three set of quantifications (proportion of insulin-positive cells co-positive for MAb1, CPE or PC2) is shown in Figure 16.

STUDY 2

Evaluation of Biological Effectiveness of Gut Insulin at Regulating Blood Glucose.

Although NOD mice naturally develop diabetes, this process normally takes months. Therefore, to rapidly destroy pancreatic β-cells so that gut K-cells would be the main insulin source, STZ, a β-cell toxin, was administered IP to age-matched transgenic NOD mice and their littermates. As shown in Figure 17A, blood glucose of both non-transgenic and transgenic mice increased following STZ administration, reaching peak levels by day 9 post-STZ treatment. Although the observed trend of lower blood glucose levels for transgenic mice compared to their non-transgenic littermates was not significant (Fig. 17A), separating the blood glucose values by sex revealed sex-differences in blood glucose regulation following STZ-treatment. Male non-transgenic and transgenic NOD mice showed a similar increase in blood glucose levels, reaching a plateau of over 30 mM by day 15 post-STZ treatment (Fig. 17B). In contrast, as shown in Figure 17C, female transgenic NOD mice overall maintained lower blood glucose levels than their non-transgenic littermates following STZ-treatment (16.0 ± 6.0 mM vs. 30.9 mM...
**Figure 14.** PC2 staining of pancreatic and duodenal tissue sections from a 6-month old female transgenic NOD mouse. Tissue sections were stained for insulin (INS; green), PC2 (red) and nuclei (DAPI; blue). The white arrow indicates an insulin-positive cell that was PC2-negative; the yellow arrow indicates an insulin-positive cell that was also PC2-positive. Images were taken at a magnification of 200x for pancreas and 100x for duodenum.
Figure 15. Quantification of insulin-positive gut cells that were co-PC2 positive along the small intestine of transgenic NOD mice. The small intestines of 6-7 month old female transgenic NOD mice were cut into 2-2.5 cm segments, paraffin embedded and sectioned. The gut sections were stained for insulin (Linco) and PC2. The percent of insulin-positive cells that were also PC2-positive was then determined and is presented in the graph for each mouse. n = 2.
Figure 16. Insulin processing quantifications along the small intestine of female transgenic NOD mice. The proportion of insulin-positive gut cells that were co-MAb1 (filled circles), co-CPE (open squares) or co-PC2 (open circles)-positive along the small intestine. Graph shown is a compilation of figures 11, 13 and 15, where data represents mean (n < 3) or mean ± SEM (n > 3).
Figure 17. Effect of streptozotocin (STZ) treatment on blood glucose levels of NOD mice (2.5 months old). Random blood glucose, measured from the tail vein with a hand held glucometer, of (A) female and male, (B) only male and (C) only female non-transgenic (NTg; open squares) and transgenic (Tg; filled circles) NOD mice following STZ treatment (180 mg/g body weight, injected intraperitoneally) on day 0. Animals were monitored daily for 1 week following STZ treatment and then twice weekly for 2 weeks. n = 2, 1, 3 and 4 for NTg female and male and Tg female and male NOD mice, respectively. Values represent mean ± SEM for n ≥ 3; mean for n < 2.
for transgenic and non-transgenic mice, respectively on day 20 post-STZ treatment). Immunostaining of pancreas sections for insulin indicated that STZ had indeed destroyed the majority of \( \beta \)-cells and that the amount of insulin-immunoreactivity remaining in the pancreas of non-transgenic (Fig. 18, top panel) and transgenic (Fig. 19, top panel) mice was comparable. As expected, no insulin-immunoreactivity was observed in the duodenum of non-transgenic mice (Fig. 18, bottom panel) while many co-insulin-immunoreactive GIP-positive cells (i.e. K-cells) were detected in the duodenum of transgenic mice (Fig. 19, bottom panel). Overall, this STZ-study suggests that insulin-producing K-cells at least protect transgenic female NOD mice from developing severe diabetes following STZ-mediated destruction of their \( \beta \)-cells.

**STUDY 3**

**Evaluation of diabetes incidence in transgenic NOD mice compared to non-transgenic littermates.**

As mentioned earlier, NOD mice normally develop overt diabetes by 4-6 months of age, with female mice showing a higher incidence than male mice (80-90% in females vs. 10-40% in males (55)). Therefore, to determine whether insulin produced from gut K-cells protects NOD mice from developing spontaneous autoimmune diabetes, the cumulative incidence of diabetes (as defined by a random blood glucose value > 20 mM) was evaluated in transgenic NOD mice and their non-transgenic littermates. As shown in Figure 20A, female transgenic NOD mice have a significantly (approximately 2.5-fold) lower incidence of diabetes than their littermates (Logrank test, \( P < 0.05 \)), where only 17.2% and 25.5% of female transgenic NOD mice were overtly diabetic at 7 and 12 months of age, respectively compared to 49.3% and 66.2% of non-transgenic littermates of the same ages. As expected, a lower incidence of diabetes was observed in the male NOD mice, with transgenic mice showing a trend of decreased incidence of diabetes compared to their non-transgenic littermates (Fig. 20B). Up to 7 months of age, male non-transgenic and transgenic NOD mice showed a similar diabetes incidence (13.4% vs.
Figure 18. Immunostaining of pancreatic and duodenal tissue sections from a 2.5-month old female non-transgenic NOD mouse with a blood glucose of 27.8 mM 20 days post-streptozotocin (STZ) treatment. Pancreas and duodenal tissues were harvested from the mouse 3 weeks post-STZ-treatment and fixed overnight with 4% paraformaldehyde, paraffin-embedded and sectioned. Tissue sections were then stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The arrow indicate a GIP-expressing K-cell. Images were taken at a magnification of 400x.
Figure 19. Immunostaining of pancreatic and duodenal tissue sections from a 2.5-month old female transgenic NOD mouse with a blood glucose of 4.7 mM 20 days post-streptozotocin (STZ) treatment. Pancreas and duodenal tissues were harvested from the mouse 3 weeks post-STZ-treatment and fixed overnight with 4% paraformaldehyde, paraffin-embedded and sectioned. Tissue sections were then stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The arrows indicate K-cells co-expressing GIP and insulin. Images were taken at a magnification of 400x.
Figure 20. Cumulative incidence of diabetes in NOD mice. Random blood glucose values of (A) male and (B) female non-transgenic (NTg; open squares) and transgenic (Tg; filled circles) NOD mice. Mice were monitored once monthly and blood glucose values were measured with a hand held glucometer from the tail vein. The number of mice evaluated at 0, 2, 4, 6, 8, 10 and 12 months are indicated below the graphs.
15.6 %, respectively at 7 months of age). However, by 8 months of age, the incidence of
diabetes increased for the non-transgenic male mice such that by 12 months of age, 38.2% of
non-transgenic male NOD mice were diabetic compared to 15.6% of transgenic male NOD
mice. Unfortunately, due to a small sample size, the difference in diabetes incidence of male
NOD mice did not reach significance. Since the greatest protective effect was observed in
females, all subsequent studies were performed on female NOD mice.

STUDY 4

Evaluation of Autoimmunity in Female NOD mice.

The greatest potential limitation of targeting insulin production to K-cells as a treatment for
diabetes is the possibility of autoimmune destruction of modified K-cells. Therefore, the next
objective was to examine the pancreas for presence/absence of autoimmunity and to compare
the duodenum of non-transgenic and transgenic mice for signs of autoimmunity against the
modified K-cells.

4.1 Assessment of insulin immunoreactive cells and lymphocyte infiltration in the
pancreas and duodenum.

To examine the progression of diabetes and confirm presence of pancreatic lymphocyte
infiltration, pancreas tissue sections from NOD mice of various ages were stained for insulin and
glucagon. As shown in Figures 21-23, transgenic NOD mice showed a wide variation in terms
of insulin content and lymphocyte infiltration (as indicated by the DAPI-positive cellular
infiltration around islets) in the pancreas. Of the 15 transgenic mice examined in this study, 1
mouse was hyperglycemic at time of sacrifice and had little or no insulin-positive islets in the
examined sections. The other 14 mice had normoglycemia at the time of sacrifice, of which 5
showed little or no lymphocyte infiltration in the pancreas and had many insulin-positive islets
(Fig. 21), 5 showed severe lymphocyte infiltration (peri-insulitis) and only had a few insulin-
positive islets per tissue section (Fig. 22), and 4 showed little or no lymphocyte infiltration but
Figure 21. Pancreatic and duodenal tissue sections from a 12-month old female transgenic NOD mouse with a random blood glucose of 5.2 mM and showing insulin staining in both pancreas and duodenum. Sections were stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The arrows indicate K-cells co-expressing GIP and insulin. Images were taken at a magnification of 200x.
Figure 22. Pancreatic and duodenal tissue sections from a 10-month old female transgenic NOD mouse with a random blood glucose of 11.3 mM and showing only little insulin staining in the pancreas and many insulin-positive cells in the duodenum. Sections were stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The white arrows indicate K-cells co-expressing GIP and insulin; the yellow arrow indicates a cell solely insulin-immunoreactive. Images were taken at a magnification of 200x for the pancreas and 100x for the duodenum.
Figure 23. Pancreatic and duodenal tissue sections from a 6-month old female transgenic NOD mouse with a random blood glucose of 6.4 mM and showing only little insulin staining in the pancreas and many insulin-positive cells in the duodenum. Sections were stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The white arrows indicate K-cells co-expressing GIP and insulin; the yellow arrows indicate solely insulin-immunoreactive cells. Images were taken at a magnification of 200x for the pancreas and 100x for the duodenum.
also had little or no insulin-positive islets in the examined tissue sections (Fig. 23). Similarly, non-transgenic NOD mice also showed a wide variation of pancreatic insulin content and lymphocyte infiltration. Of the 10 non-transgenic mice examined, 2 mice were hyperglycemia at time of sacrifice and showed little or no insulin-positive islets in the pancreas. The other 8 mice had normoglycemia at time of harvest, of which 2 showed little or no lymphocyte infiltration in the pancreas with many insulin-positive islets, 2 showed a mix of islets with and without peri-insulitis in the pancreas with many insulin-positive islets (Fig. 24), 2 showed severe peri-insulitis in the pancreas, and 1 showed little lymphocyte infiltration as well as little insulin staining in the pancreas.

In contrast to the observed variation in the pancreas, all transgenic NOD mice showed many insulin-positive K-cells in the duodenum, regardless of pancreas insulin content (Fig. 21-23). As expected, non-transgenic NOD mice showed no insulin immunoreactivity in the duodenum (Fig. 24). In all mice duodenal sections examined, no detectable collection of DAPI-positive cellular accumulation was observed near insulin-positive K-cells.

4.2 Gut morphological comparison of transgenic NOD mice and their littermates.

As shown in the series of insulin/GIP immunofluorescence images of the duodenum, (Fig. 21-24), unlike the clusters of insulin-producing β-cells in the pancreatic islets, K-cells are dispersed throughout the gut epithelium. Therefore, while presence of autoimmunity in the pancreas is easily detected by the massive infiltration of leukocytes that are recruited to destroy the clusters of β-cells, as indicated by the accumulation of DAPI-positive cells around islets, autoimmune targeting of insulin-producing K-cells is not expected to elicit such a concentrated immune response. Consequently, more sensitive measures were needed to evaluate whether modified insulin-producing K-cells of transgenic NOD mice are targeted by the immune system. Thus, the next objective of this study was to examine the duodenum for signs of inflammation and/or K-cell destruction.
Figure 24. Pancreatic and duodenal tissue sections from a 6-month old female non-transgenic NOD mouse with a random blood glucose of 8.2 mM and showing insulin staining in the pancreas and no insulin staining in the duodenum. Sections were stained for insulin (INS; green), glucagon (GLUC; red; pancreas) or GIP (red; duodenum) and nuclei (DAPI; blue). The arrows indicate GIP-expressing K-cells. Images were taken at a magnification of 200x.
4.2.1 Evaluating gut inflammation through villi and crypt measures.

Pancreas and duodenal sections were stained with H&E and duodenal villus and crypt lengths were measured. Similar to the fluorescent images of the pancreas shown in Figures 21-24, H&E-stained pancreas sections from non-transgenic and transgenic mice showed a mix of no lymphocyte infiltration and peri-insulitis (Fig. 25, top panel). No apparent visual differences in morphology or pathology (such as villi atrophy) were observed between H&E-stained duodenal sections from non-transgenic and transgenic mice (Fig. 25, bottom panel). There was also no difference between the two genotypes with respect to duodenal villus (non-transgenic: 625.7 ± 29.7 μm vs. transgenic: 671.7 ± 35.5 μm), crypt (non-transgenic: 134.1 ± 7.8 μm vs. transgenic: 143.3 ± 5.0 μm) or total crypt-villus (non-transgenic: 759.4 ± 32.0 μm vs. transgenic: 822.1 ± 36.7 μm) lengths (Fig. 26A) or villus to crypt ratios (non-transgenic: 4.9 ± 0.5 vs. transgenic: 5.1 ± 0.3; Fig. 26B).

4.2.2 GIP-positive cell location in epithelium

If insulin-producing K-cells are destroyed by the autoimmune system, then a difference in K-cell location along the crypt-villus axis was expected between non-transgenic and transgenic NOD mice. More specifically, if K-cells were not targeted and destroyed in transgenic mice, then the distribution of K-cells along the crypt-villus axis was expected to be fairly even and similar between non-transgenic and transgenic mice (Figure 27A). In contrast, if modified K-cells were destroyed by the immune system, then it was expected that the distribution of K-cells in the gut of transgenic mice would be negatively shifted (and thus predominantly restricted to the lower portion of the villus) compared to that of non-transgenic mice (Figure 27B). When the location of duodenal GIP-positive cells of non-transgenic and transgenic mice was determined, the resulting scatter plots, shown in Figure 27C, resembled that of the no K-cell destruction situation depicted in Figure 27A. The location of GIP-positive cells in transgenic and non-transgenic mice duodenum averaged 50.4 ± 1.8% (ranged from 2.3 to 98.9%) and 55.5 ± 1.3%
Figure 25. Representative images of pancreatic and duodenal tissue sections from female NOD mice stained with hematoxylin and eosin (H&E). Pancreas and duodenal sections from non-transgenic (NTg) and transgenic (Tg) female mice were stained with H&E. The arrows indicate islets. Images taken at a magnification of: 200X, 400X, 100X and 200X for the pancreas images, from left to right; and 100x for duodenal images.
Figure 26. Villus and crypt measures of duodenum from female NOD mice. Villus, crypt and villus-crypt lengths (A) and villus to crypt ratio (B) of duodenum from non-transgenic (NTg, n=10, open bars) and transgenic (Tg, n=12, filled bars) mice were determined from hematoxylin and eosin stained duodenal sections using the OpenLab software to measure lengths. Bars represent mean ± SEM.
Figure 27. Location of GIP-positive cells along the crypt-villus axis. (A) and (B) represent hypothetical scatter plots of what would be expected if there was no destruction of insulin-producing K-cells (A) and if there was destruction of insulin-producing K-cells (B). (C) Comparison of the location of GIP-positive cells in the duodenum of non-transgenic (NTg, n=10, open squares) and transgenic (Tg, n=12, filled circles) NOD mice. (D) Comparison of the proportion of GIP-positive cells located in the bottom (0-33.3%), middle (33.4-66.6%) and top third (66.7-100%) along the crypt-villus axis in NTg (open bars) and Tg (filled bars) NOD mice. For (A), (B) and (C), each point represents one GIP-positive cell and red lines represent Mean ± SD. For D, bars represent mean ± SEM. * P < 0.05 (NTg vs. Tg).
(ranged from 1.4 to 99.8%) of crypt-villus length, respectively, suggesting that K-cells in the duodenum of transgenic mice as a group were located slightly but significantly closer to the villus tip than K-cells in the duodenum of non-transgenic mice ($P < 0.05$). The proportion of K-cells in the bottom (0-33.3%), middle (33.4-66.6%) and top (66.7-100%) portion of the crypt-villus axis was not different between non-transgenic (29.9 ± 4.5%, 43.4 ± 5.5% and 29.2 ± 4.1%, respectively) and transgenic (23.7 ± 1.6%, 40.1 ± 2.7% and 36.2 ± 3.1%, respectively) mice duodenal epithelium (Fig. 27D). Overall, duodenal K-cells of transgenic and non-transgenic mice were similarly distributed along the crypt-villus axis.

4.3 Examining gut K-cell autoimmunity by comparing number of leukocytes in the duodenum.

The previous studies showed that there was no detectable difference in duodenal morphology between non-transgenic and transgenic NOD mice, no obvious collection of leukocytes around insulin-producing K-cells, nor destruction of insulin-producing K-cells. However, with the dispersed and migratory (along a given villus) nature of K-cells, perhaps insulin-producing K-cells do trigger an immune response but have changed locations along the villus before being destroyed. Therefore the goal of this study was to determine whether there were differences in leukocyte numbers in the duodenum of non-transgenic and transgenic mice.

4.3.1 Total leukocyte quantification in the duodenum

To determine whether insulin-producing K-cells elicit an autoimmune response, duodenal sections of mice previously shown to have lymphocyte infiltration in the pancreas were stained for the general leukocyte marker, CD45. Representative images of pancreas and duodenal sections stained for CD45 and insulin (pancreas) or GIP (duodenum) of non-transgenic and transgenic mice are shown in Figures 28 and 29, respectively. The number of CD45-positive cells were counted and expressed as the number of CD45-positive cells per 100 μm villus measured. As suggested by Figure 30A, there was no statistical difference in the number of
Figure 28. Pancreatic and duodenal tissue sections from a 6-month old female non-transgenic NOD mouse showing peri-insulitis in the pancreas. Sections were stained for insulin (INS; green; pancreas) or GIP (green; duodenum), CD45 (red) and nuclei (DAPI; blue). The duodenal image on the right is an overlay of GIP, CD45, DAPI and dark field (DF) images. The arrow indicates a GIP-positive cell. Images were taken at a magnification of 200x.
Figure 29. Pancreatic and duodenal tissue sections from a 12-month old female transgenic NOD mouse showing peri-insulitis in the pancreas. Sections were stained for insulin (INS; green; pancreas) or GIP (green; duodenum), CD45 (red) and nuclei (DAPI; blue). The duodenal image on the right is an overlay of GIP, CD45, DAPI and dark field (DF) images. The arrows indicate GIP-positive cells. Images were taken at a magnification of 200x.
Figure 30. Quantification of leukocytes and intraepithelial lymphocytes (IELs) in the duodenum of female NOD mice. The number of (A) CD45 (general leukocyte marker)-positive cells and (B) CD3-positive IELs detected in the duodenum of non-transgenic (NTg, n=4, open bar) and transgenic (Tg, n=4, filled bar) female NOD mice. The number of CD45-positive cells were counted and normalized to villus length, measured with the OpenLab software while the number of CD3-positive IELs were counted and normalized to the number of epithelial cells. Bars represent mean ± SEM.
total leukocytes in the duodenum of non-transgenic and transgenic NOD mice (7.3 ± 0.6 and 7.7 ± 0.6 CD45-positive cells/100μm villus, respectively).

4.3.2 Intraepithelial T cell quantification in the duodenum

Since T cells are known to play a major role in the destruction of pancreatic islets, the number of T cells in the duodenum was examined. Figures 31 and 32 show representative images of pancreas and duodenal sections stained for CD3 and insulin (pancreas) or GIP (duodenum) from non-transgenic and transgenic NOD mice, respectively. The number of duodenal intraepithelial T cells, a measure that is commonly used to detect gut pathology in gastrointestinal diseases, was then counted and normalized to the number of epithelial cells. As shown in Figure 30B, there was no statistical difference in intraepithelial T cell density between non-transgenic and transgenic NOD mice (8.9 ± 1.3 and 7.9 ± 0.4 intraepithelial T cells/100 epithelial cells, respectively).
Figure 31. Pancreatic and duodenal tissue sections from a 7-month old female non-transgenic NOD mouse showing peri-insulitis in the pancreas. Sections were stained for insulin (INS; green; pancreas) or GIP (green; duodenum), CD3 (red) and nuclei (DAPI; blue). The duodenal image on the right is an overlay of GIP, CD3, DAPI and dark field (DF) images. The arrow indicates a GIP-positive cell. Images were taken at a magnification of 100x for the pancreas and 200x for the duodenum.
Figure 32. Pancreatic and duodenal tissue sections from a 12-month old female transgenic NOD mouse showing peri-insulitis in the pancreas. Sections were stained for insulin (INS; green; pancreas) or GIP (green; duodenum), CD3 (red) and nuclei (DAPI; blue). The duodenal image on the right is an overlay of GIP, CD3, DAPI and dark field (DF) images. The arrow indicates a GIP-positive cell. Images were taken at a magnification of 200x.
DISCUSSION

Targeting Insulin Expression to Gut K-cells of NOD Mice

T1DM is a chronic metabolic disease characterized by insulin deficiency resulting from T cell mediated destruction of insulin-producing pancreatic \( \beta \)-cells. Lacking endogenous insulin, patients with T1DM rely on exogenous insulin to maintain their blood glucose levels. However, even with intensive insulin therapy, optimal blood glucose levels are rarely achieved, leaving patients at increased risks for episodes of hypoglycemia and long-term health complications associated with hyperglycemia. Gene therapy offers an attractive mode of insulin delivery, where endogenous production and release of insulin within the body can be achieved while avoiding the problems of donor shortage, life-long immunosuppression and ultimate reversal to insulin-dependence associated with pancreas and islet transplantations. While targeting gut K-cells has been previously shown to protect mice from developing STZ-induced diabetes (44), autoimmunity against these modified K-cells may be a potential limitation of this approach. Therefore, this project aimed to investigate this possibility, using the NOD mouse model of T1DM. Unlike Cheung et al. (44), who used a human insulin-expressing transgene (rGIP/hlNS), in this study, transgenic NOD mice were generated by introducing into NOD embryos a mouse insulin-expressing transgene (rGIP/mINS; Fig. 3A). The use of human insulin allows for the differentiation of native mouse insulin from insulin expressed off of the transgene in plasma samples as well as in gene expression studies (e.g. northern blot or reverse-transcriptase polymerase chain reaction [RT-PCR]). Thus with the rGIP/hlNS transgenic mice, pancreatic insulin content could be approximated by measuring plasma mouse insulin level instead of sacrificing mice and staining their pancreas for insulin. However, considering that even single amino acids can change the structure of epitopes and thus potential recognition sites of immune components, this study used mouse insulin. Mouse insulin II was selected as opposed to
mouse insulin I since, as discussed earlier, mouse insulin II compared to insulin I has been found to be protective against diabetes (85).

Although the transgene was introduced into mice at the embryonic level and thus should be present in all cells of transgenic mice not all cells express insulin. In fact, as previously observed in the rGIP/hlNS transgenic mice employing the same GIP promoter sequence, insulin expression driven by the GIP promoter was limited to GIP-producing tissues, namely the stomach and duodenum (44). Staining the duodenum for insulin and GIP showed that insulin expression is restricted to GIP-expressing cells (Fig. 3B and 3C), in other words, to K-cells, which contain the required collection of transcription factors to bind the GIP promoter and drive insulin expression.

**Characterization of Insulin Protein Expression in Modified Gut K-cells**

In this study, endocrine cells producing insulin (and therefore GIP) and GLP-1 were found in all regions of the small intestine of transgenic NOD mice. By double immunohistochemistry, the coexistence (i.e. colocalization) of insulin and GLP-1 in the same cells (Fig. 8) in different regions of the small intestine was assessed. In the proximal small intestine, many cells that were exclusively insulin-immunoreactive were observed while many exclusively GLP-1-immunoreactive cells were observed in the distal ileum (Fig. 9). The existence of GIP (as suggested by the presence of insulin in transgenic mice) and GLP-1 colocalization has been previously observed in porcine, human and rat small intestines (121, 122). In fact, by combining immunohistochemistry for GIP with in situ hybridization (ISH) for proglucagon (the primary translation product of the GLP-1 encoding gene in the gut), Mortensen et al. (121) also showed colocalization of GIP-immunoreactivity with proglucagon expression. The existence of solely insulin-producing gut cells (i.e. K-cells) and of insulin- and GLP-1-producing gut cells (i.e. L/K-cells (122)) indicates that GIP-expressing cells are not all the same but are instead heterogeneous, suggesting the possibility of differential expression of processing enzymes.
within the population of GIP-expressing gut cells (as was observed in the quantification studies discussed below).

Similar to many peptide hormones and neurotransmitters, proinsulin is processed post-translationally. The conversion of proinsulin to insulin mainly takes place in immature secretory granules (123) and occurs by a branched pathway (Fig. 4). The prohormone convertases PC1/3 and PC2 cleave proinsulin at Arg\(^{32}\)-Arg\(^{33}\) (BC junction) and Arg\(^{65}\)-Gly\(^{66}\) (AC junction) resulting in the formation of split-Arg\(^{32}/\)Glu\(^{33}\)-proinsulin and split-Arg\(^{65}/\)Gly\(^{66}\)-proinsulin respectively in a ratio of about 3:1 (124-126). Des-Arg\(^{31}\), Arg\(^{32}\)-proinsulin and des-Lys\(^{64}\), Arg\(^{65}\)-proinsulin are then formed following the removal of the exposed C-terminal basic residues by CPE (127). Cleavage of either intermediate by the corresponding prohormone convertase (PC2 and PC1/3, respectively) and subsequent removal of the exposed basic C-terminal amino acids yields insulin and C-peptide (117, 127).

The various insulin intermediates have different receptor binding affinity and biological activity. In IM-9 lymphocytes, freshly isolated rat adipocytes and purified rat liver membranes, Peavy et al. (119) found that human proinsulin had ~1% receptor binding affinity of insulin, while modifications of proinsulin near the AC junction to various split- and des-proinsulin forms (i.e. split-Arg\(^{65}/\)Gly\(^{66}\)-proinsulin, des-Ala\(^{57}\)-Arg\(^{65}\)-proinsulin, des-Lys\(^{64}/\)Arg\(^{65}\)-proinsulin and des-Glu\(^{33}\)-Leu\(^{56}\)-proinsulin) and at the BC junction to split-Arg\(^{32}/\)Glu\(^{33}\)-proinsulin or des-Arg\(^{31}/\)Arg\(^{32}\)-proinsulin resulted in 11-27 and 5 fold increases in receptor affinity compared to human proinsulin, respectively. These trends were also reflected in the relative biological activity (as measured by incorporation of [2-\(^3\)H]glucose into total cell lipid) in isolated rat adipocytes (119).

In terms of the effect of removing the two arginine residues from the C-terminus end of the insulin B-chain on biological activity, there have been mixed findings. In 1973, Yu and Kitbachi reported that porcine diarginylinulin had only 40% biological activity compared to insulin (120). However, subsequent studies by Peavy et al. (119) found that retention of the dibasic residues in split-Arg\(^{32}/\)Glu\(^{33}\)-proinsulin and split-Arg\(^{65}/\)Gly\(^{68}\)-proinsulin had little effect on biological activity
compared to their respective desdipeptide forms. Similarly, Zeuzem et al. (128) found that biosynthetic human diarginylinsulin had similar insulin receptor binding (association/dissociation rate constants and steady-state competition displacement curves) and biological activity (ability to stimulate D-glucose transport and antilipolytic activity) in human adipocytes compared to native insulin. Explanations that have been proposed for the apparently contradictory findings of Yu and Kitbachi (120) compared to Peavy et al. (119) and Zeuzem et al. (128) include: 1) the potential cross contamination with proinsulin of partially cleaved proinsulin in the earliest studies by Yu and Kitabchi; and 2) the possibility that the single amino acid difference between porcine and human insulin at position B30 (Porcine Ala$^{B30}$ vs. Human Thr$^{B30}$) results in a difference in biological activity when the two arginine residues remain attached despite no differences in activity between fully processed porcine insulin and human insulin (128). Overall these studies suggest that the degree of insulin processing and even single amino acid differences affect receptor binding affinity and biological activity of insulin and its processing intermediates. In addition, the fact that an antibody specific to C-terminus of the human insulin B-chain (MAb1) has significantly reduced cross-reactivity to human mono- and diarginylinsulin indicates that even single amino acid sequence differences results in structural changes in insulin that are detected by antibodies, suggesting that even minor differences in insulin sequence may elicit different immune responses. Therefore, this thesis also investigated whether modified gut K-cells contained processed insulin and the necessary processing enzymes.

The antibody MAb1 was used to examine processing at the BC junction. Quantification of gut sections co-stained with a relatively nondiscriminatory insulin antibody (i.e. one that recognizes insulin and its precursor forms) and mAB1 (Fig. 10) showed that in the duodenum to mid-ileum of transgenic NOD mice, only 20.1 ± 1.8% of insulin-positive gut cells contained detectable amounts of insulin that had been processed at the BC junction (Fig. 11). Considering that MAb1 cross-reactivity decreases significantly when the two arginine residues
are still attached to the B-chain or when Arg\textsuperscript{32}–Arg\textsuperscript{33} remains intact (see Fig. 4), this observation suggests that there is insufficient quantity and/or activity of PC1/3 and/or CPE.

Staining for insulin and PC1/3 demonstrated that most insulin-immunoreactive gut cells were also PC1/3 positive (data not shown). In fact, Ugleholdt \textit{et al.} (129) observed PC1/3 immunoreactivity in all GIP-immunoreactive cells of the murine intestine. Using PC1/3 and PC2 null mice to examine proGIP processing, they also found that while proGIP processing in PC2-deficient animals was normal, processing to GIP was severely impaired in PC1/3-deficient mice (129). Furthermore, \textit{in vitro} studies with various cell lines using adenovirus-mediated over-expression of preproGIP and various processing enzymes found that GIP was produced in cell lines naturally expressing (ArT-20) or made to express PC1/3 with adenovirus (GH\textsubscript{4}) and little or no PC2. Cell lines only expressing PC2 (α-TC1.9; and GH\textsubscript{4} cells infected with adenovirus expressing PC2) showed that although processing of proGIP by PC2 produced GIP, other fragments not found in intestinal extracts were also produced (129). Based on these findings, Ugleholdt \textit{et al.} (129) conclude that PC1/3 is necessary and sufficient for processing of proGIP to GIP and that although able to cleave proGIP, PC2 is not involved with intestinal proGIP processing nor is it found in GIP-expressing intestinal cells. Therefore, in light of the importance of PC1/3 and its reported presence in all GIP-immunoreactive cells, PC1/3 is unlikely the reason for the observed low amount of co-MAb1 staining in the insulin-positive gut cells of transgenic NOD mice. Instead, as supported by the presence of CPE immunoreactivity in only 39.3% of insulin immunoreactive gut cells (duodenum to distal jejenum, Fig. 12 and 13) and the similarity between the proportion of insulin-positive cells showing immunoreactivity for BC junction-processed insulin and for CPE along the length of small intestine (Fig. 16), the lack of CPE is the probable reason for incomplete processing of proinsulin at the BC junction. Based on the MAb1 and CPE quantifications, it appears that the majority of insulin-producing gut cells contain diarginylinsulin or split-Arg\textsuperscript{32}/Glu\textsuperscript{33}-proinsulin (refer to Fig. 4).
To investigate proinsulin processing at the AC junction, the presence of the preferred processing enzyme of this junction, PC2 in insulin-producing gut cells was evaluated. Quantifying insulin-positive gut cells revealed that only a small proportion (around 28%) of these cells contained detectable amounts of PC2 (Fig. 14 and 15), indicating that proinsulin processing at the AC junction was suboptimal in the majority of insulin-containing gut cells. The observed existence of a population of insulin-positive, and thus GIP-positive, intestinal cells that were co-immunoreactive for PC2 in the transgenic NOD mice is in contrast to the previously reported lack of such co-staining (129). A possible explanation for this contradictory finding may be the fact that Ugleholdt et al. (129) used a GIP antibody (3.65 H) specific to the C-terminal of PC1/3 processed-GIP. Co-staining gut sections with the 3.65 H GIP antibody and another GIP antibody that recognizes a central region of the GIP molecule suggests the existence of different populations of GIP-expressing cells containing differentially processed GIP and thus a different collection of processing enzymes (Fig. 6). Therefore the PC2 quantification indicates that a subset of insulin-producing K-cells contains both PC1/3 and PC2 processing enzymes.

Although PC2 is required for efficient processing of proinsulin to insulin, Furuta et al. (130) found that compared to PC1/3, PC2 is less important in proinsulin processing. In contrast to the severe block in proinsulin conversion to insulin in PC1/3 null mice (131), PC2 null mice, which completely lack active PC2, only exhibited partial defects in proinsulin processing, showing a 3-fold decrease in the rate of proinsulin conversion to insulin and a 4 to 5-fold increase in the level of des-31,32 proinsulin (130). The subcellular localization of proinsulin is also altered in mutant mice; in contrast to the localization of proinsulin to the Golgi apparatus and maturing secretory granules in wild type β-cells, proinsulin immunostaining was also observed in the majority of secretory granules of mutant β-cells (130). Furthermore, the secretory granules failed to form the typical dense crystalline granule cores and instead appeared pale and homogenous in electron microscopic images (130). However, despite these processing deficiencies resulting from a lack of active PC2, proinsulin was still efficiently sorted into the regulated secretory
pathway and two-thirds of proinsulin was processed to insulin in maturing secretory granules of β-cells (130). Thus while PC1/3 preferentially cleaves at the BC junction of proinsulin (132), these findings support the ability of PC1/3 to cleave at both junctions and convert proinsulin into insulin (130, 133). Therefore, although PC2 gut quantification in this thesis found that the majority of insulin-producing K-cells lacked PC2, around two thirds of proinsulin produced in the PC2-deficient insulin-producing K-cells should have been properly sorted into the regulated secretory pathway and cleaved at both junctions.

Overall, these quantifications suggest that the majority of insulin-producing K-cells of transgenic NOD mice lack one or more of the required proinsulin processing enzymes and thus secrete incompletely processed insulin. In fact, of the insulin-producing gut K-cells, around 25% probably produced insulin with an intact AC junction while around 60% produced insulin with the two arginine residues still attached to the C-terminus of the B-chain. In light of the studies showing decreased insulin receptor affinity and biological activity of insulin intermediates (119, 120, 128), the proinsulin processing defects of K-cells indicated by these quantification studies may partly account for the incomplete protection of transgenic mice from both spontaneous and STZ-induced diabetes, as discussed below.

Effectiveness of Insulin from Gut K-cells at Protecting Transgenic NOD Mice from Developing STZ-induced Diabetes

Used commonly to induce diabetes in rodents, STZ has been suggested to mediate its toxicity effects on β-cells via DNA methylation and damage, poly ADP ribose polymerase (PARP) activation (134), FAS expression upregulation (135), and O-GlcNAc-selective N-acetyl-β-D-glucosaminidase, the enzyme responsible for removing O-N-Acetylglucosamine (O-GlcNAc) from proteins, inhibition (136). In this study, blood glucose levels of NOD mice increased following STZ-treatment. Although as a whole, transgenic mice only showed a trend of lower blood glucose values following STZ-treatment (Fig. 17A), there appeared to be a sex difference.
When the blood glucose values were analyzed by sex, this study found that male transgenic NOD mice were not protected from developing diabetes, as indicated by the nearly superimposable blood glucose profiles of male non-transgenic and transgenic NOD mice, where elevated blood glucose values of over 30 mM were measured in both groups of mice by day 15 (Fig. 17B). In contrast, STZ-treated female transgenic NOD mice were able to maintain lower blood glucose levels compared to their non-transgenic littermates (Fig. 17C) despite showing similar amount of remnant pancreatic insulin immunoreactivity (Fig. 18 and 19, respectively).

Although this study should be repeated with an increased sample size, these results suggest that insulin-producing K-cells were able to protect transgenic female NOD mice from developing severe diabetes. However, the fact that transgenic mice, especially male mice, showed increased blood glucose following STZ-treatment indicates that insulin produced from gut K-cells is insufficient in quantity and/or biological activity (perhaps due to incomplete proinsulin processing, as discussed above) to properly regulate blood glucose levels.

The observed difference between female and male transgenic NOD mice may in part reflect a sex difference in insulin sensitivity. Female Wistar rats were found to be 4.5-fold more sensitive to insulin and developed less severe insulin resistance following chronic (5 weeks) insulin treatment (2U/day) compared to their male counterparts (137). The greater sensitivity to insulin observed in female rodents is perhaps partly due to estrogen. Post-menopausal women develop insulin resistance and are at an increased risk for developing T2DM, the incidence of which decreases with hormone replacement therapy (138). Similarly, in animal models of T2DM, females are protected against the disease unless they are ovariectomised and males show reversal of diabetes following 17β-estradiol perfusion (138). The actions of estradiol have been suggested to include: regulation of the amount of white adipose tissue (139); stimulation of liver fatty acid metabolism and suppression of hepatic glucose production; and protection of pancreatic β-cell function and survival and insulin secretion under conditions of oxidative stress (138). In estradiol-deficient (aromatase knockout [ArKO]) and estradiol-resistant (estrogen
receptor-α knockout [αERKO]) male and female mice, the observed increase in β-cell apoptosis and susceptibility to STZ-induced diabetes were reversed with estradiol treatment (140).

In contrast to the insulin-sensitizing effects of estradiol, androgens like testosterone, have been found to decrease insulin sensitivity by altering plasma levels of adiponectin, an adipose-specific secretory peptide with antidiabetic and antiatherogenic properties (141). Nishizawa et al. (141) found that plasma adiponectin levels are significantly lower in men compared to women but no different in pre- and postmenopausal women. In mice, ovariectomy had no affect on plasma adiponectin levels, while castration resulted in significant improvements in insulin sensitivity associated with higher plasma levels of adiponectin (141). Furthermore, testosterone treatment of mice and adipocyte cells (3T3-L1 cells) reduced adiponectin plasma concentration and secretion, respectively (141). In light of these findings, the higher level of estrogen in female mice likely helped protect female transgenic mice against the diabetogenic effects of STZ while the higher levels of androgens in male mice made male transgenic mice more susceptible to STZ-induced diabetes.

Incidence of Spontaneous Diabetes in Transgenic NOD Mice with Insulin-Expressing K-cells

In this study, long-term monthly tracking of blood glucose levels of transgenic NOD mice and their non-transgenic littermates showed that female transgenic mice had a lower incidence of diabetes compared to non-transgenic female NOD mice (Fig. 20B). Male NOD transgenic mice similarly showed a trend of decreased diabetes incidence compared to their non-transgenic littermates (Fig. 20A). The increasing disease incidence observed with age in both sexes of NOD mice likely reflects two factors. First, the large increase observed between 6 and 10 months of age, especially in non-transgenic mice, is probably due to the autoimmune mediated destruction of pancreatic β-cells. Without an alternative source for insulin, blood glucose levels rise and overt diabetes (indicated by polyuria and decreased body weight) develops in non-
transgenic mice as their immune system destroys their β-cells. The immune system similarly targets and destroys the β-cells of transgenic mice (Fig. 21-23, 25, 29 and 32, top panels). However, with an alternative source for insulin, namely their gut K-cells (Fig. 21-23, bottom panel), significantly more transgenic mice are able to maintain their blood glucose levels despite severe elimination of β-cells. Secondly, aging has been associated with decreased insulin sensitivity (142, 143). Thus as the NOD mice aged and became less sensitive to insulin, defects in quantity and/or quality of gut insulin became more apparent, as manifested by the development of diabetes in older female transgenic NOD mice. The lack of a similar increase in diabetes incidence in older male transgenic mice despite age-related decrease in insulin sensitivity likely reflects the countering effect of increased insulin sensitivity resulting from decreased testosterone secretion associated with aging (144).

The difference in diabetes incidence between male and female NOD mice has also been partly attributed to differences in leptin levels between the two sexes. Leptin, a 16-kDa product of the obese gene (145), is a hormone that is produced in and released from white adipose tissue and normally circulates at a level proportional to total adipose mass (146). Besides being higher in female NOD mice compared to their male counterparts, leptin levels, independent of body weight and food intake, surge 5-10 fold prior to and then decrease during the clinical onset diabetes (147). Administration of leptin to young female NOD mice accelerates the onset and increases the severity of diabetes (147). In addition to its effect on food intake, energy metabolism (148, 149), and insulin secretion (150-153) and sensitivity (154), leptin has also been shown to influence T cell function (155) and survival (156). It has been suggested that by favoring Th1 differentiation and interleukin-2 (IL-2) secretion and upregulating Bcl-2 expression, leptin promotes early inflammatory infiltration of pancreatic islets and accelerates the onset and progression of T1DM (147, 155, 156). The involvement of leptin in the development of T1DM is supported by the lower incidence of autoimmune diabetes observed in NOD mice with a mutation in the extracellular domain of the leptin receptor (NOD-Lper^{db-Sj}) (157). Although these
mice develop early onset hyperglycemia, all obese females and a subset of obese males continue to gain weight and eventually show spontaneous diabetes remission associated with little insulitis and β-cell hyperplasia (157). While plasma corticosterone level, percentage of splenic CD4+ or CD8+ T cells and in vitro T cell function were not different between mutant NOD-Lper^{db-5J} and wild-type NOD mice, splenocytes from hyperglycemic mutant donors did not transfer diabetes to recombination-activating gene (RAG; encodes for RAG1 and 2, which are proteins necessary for immunoglobulin and TCR gene recombination)-null NOD (NOD-Rag^{−/−}) recipients. Mutant NOD-Lper^{db-5J} mice also had reduced percentage and activation of insulin and islet specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD8+ T effector clonotypes (158). In addition, mutant NOD-Lper^{db-5J} mice displayed hyperinsulinemia, which is likely due to decreased leptin induced suppression of insulin secretion (150-153). The observed hyperinsulinemia has been suggested to be largely responsible for the decreased T1DM incidence in mutant NOD mice (158) since hyperinsulinemia destroys the insulin gradient that has been proposed to be necessary for macrophage extravasation during the earliest stages of insulitis initiation (159).

The heterogeneous development of clinical diabetes among transgenic mice of the same age points to possible differences in transgene copy number. Since insertion of transgene into embryonic mouse genome is a random event with embryonic injection, multiple copies of the transgene could have been inserted into the genome, of which one or more copies could insert into multiple chromosomes. As such, the offspring of transgenic mice with multiple chromosomes containing the transgene may inherit different number of transgene copies. Consequently, such offsprings would have different levels of transgene expression and thus insulin protein level in the gut.

The observed lower cumulative diabetes incidence in the female NOD non-transgenic mice (66% at 12 months) than the commonly reported incidence of 80-90% for female NOD mice probably reflects the effect of environment on diabetes incidence, where “dirty” colonies,
compared to “clean” colonies (strict specific pathogen-free conditions), are often associated with lower disease incidence, presumably due to increased stimulation of the immune system by infections (160). However, overall this study suggests that female mice expressing the rGIP/mINS transgene, and thus bearing insulin-producing K-cells, are partially protected from developing spontaneous diabetes.

**Autoimmune Diabetes Progression**

T1DM disease progression is characterized by progressive destruction of pancreatic islet β-cells. In this study, pancreas tissue sections from mice of different ages showed varying degrees of disease progression as indicated by their blood glucose levels and differences in pancreatic insulin staining (Fig. 21-24). For non-transgenic mice, blood glucose levels matched pancreatic insulin-immunoreactivity fairly closely, where hyperglycemia was associated with a little or lack of insulin-immunoreactivity. In contrast, blood glucose values of transgenic mice often did not reflect insulin content in the pancreas. In fact, a third of the transgenic female mice with normoglycemia showed little or no insulin immunoreactivity in the pancreas sections examined (Fig. 22, 23). In contrast to the large variation in insulin content of the pancreas, all transgenic mice showed many insulin-positive K-cells in the duodenum, regardless of blood glucose and pancreas insulin content (Fig. 21-23). Thus insulin-producing gut K-cells were still present following immune-mediated destruction of pancreatic β-cells. The unexpected observation of cells showing insulin-immunoreactivity but no GIP-immunoreactivity likely reflects the different populations of GIP-expressing gut cells discussed above. More specifically, considering that a subpopulation of GIP-expressing cells contains PC2, which can cleave GIP at a PC2 site within the full length GIP(1-42) molecule, the C-terminus-specific GIP antibody used in this study would have missed this subpopulation of cells expressing a C-terminally-truncated GIP (1-30).
Overall, the presence of numerous insulin-producing K-cells and relatively normal blood glucose levels in mice with little or no pancreatic insulin suggests that the blood glucose levels of these mice were mainly maintained by insulin from the gut. However, it should be noted that since only a very limited number of sections were sampled from each pancreas and that these sections were not systematically selected, it is possible that the sections examined were not representative of the entire pancreas and thus insulin might have been present in other areas of the pancreas that was missed in this study. Therefore, at this time, the possibility that residual pancreatic insulin contributed to the normoglycemia observed in many transgenic mice cannot be excluded. Thus a more detailed and systematic analysis of the pancreases of NOD mice is needed to accurately determine the degree of β-cell destruction.

Regardless of the actual status of insulin content of the pancreas, the abundance of insulin immunoreactivity observed in the duodenum suggests that the modified K-cells are not destroyed by the same autoimmune process that destroys β-cells. However, the rapid turnover of gut epithelial cells means that simply looking for the presence of insulin staining in the gut is not sufficient to rule out autoimmune targeting and destruction of the gut K-cells. Therefore, this study next examined the duodenum for any histopathological signs of immune attack of the insulin-producing K-cells.

Constantly in contact with food antigens and the gut flora, maintaining the integrity of the gut epithelium, which involves continuous epithelial cell turnover, is necessary for local defense against potential pathogens that enter the host through the intestine (161). Thus with a continual replacement of gut epithelial cells, it is expected that if the insulin-producing K-cells trigger the autoimmune system, then chronic inflammation of the gut would ensue, leading to morphological changes. One common characteristic of many disorders/diseases of the small intestine, such as celiac disease (162) and tropical sprue (163), is the concurrent shortening of villi and lengthening of crypts, and thus decreased villus to crypt ratio, and increased crypt proliferation and hypertrophy in response to enterocyte damage. In this study, no gross
morphological differences were observed between female transgenic NOD mice and their non-transgenic littermates (Fig. 25) nor were there any differences in villus and crypt lengths, epithelial thickness as determined by the sum of the villus and crypt lengths (Fig. 26A), or villus to crypt ratio between the two genotypes (Fig. 26B). The observed villus to crypt ratios for non-transgenic and transgenic female NOD mice are consistent with the reported ratio of 4:1 to 5:1 for normal villi (164). Thus, insulin-production in K-cells does not appear to elicit gross pathological changes to or destruction of the gut epithelium. However, in light of the disperse nature of K-cells, immune-mediated destruction of these cells may not elicit massive morphological changes, requiring a more detailed examination of the K-cells themselves.

Derived from gut stem cells in the crypts of Lieberkuhn, gut enterocytes, including K-cells, differentiate in the crypt and then migrate up the villus (90). Two to 7 days after differentiation, epithelial cells reach the villus tip where they are subsequently sloughed off into the gut lumen (90). With this continual renewal, migration and sloughing off of epithelial cells, K-cells are normally found dispersed throughout villus along the crypt-villus axis of the gut epithelium (Fig. 27A). In contrast, if modified K-cells are targeted and destroyed by the immune system, then it was expected that K-cells would be destroyed before being able to migrate up the villus. Consequently, K-cells would be predominantly found in the lower portion of the crypt-villus axis (Fig. 27B). In this study, no such alteration in K-cell location was observed in the transgenic mice compared to their non-transgenic littermates (Fig. 27C). In fact, the proportion of K-cells in the top, middle and bottom portions of the villi were similar between the two genotypes (Fig. 27D) and the average (mean) location of K-cells in the duodenum of transgenic mice was slightly higher (statistically significant) along the crypt-villus axis than that in the duodenum of non-transgenic mice. Therefore, the lack of a negative shift in the distribution of duodenal K-cells of transgenic NOD mice compared to their non-transgenic littermates suggests that there was no increased destruction of insulin-producing K-cells. However, despite these results, the
possibility of increased recognition and leukocyte infiltration of gut epithelium warranted further investigation.

Unlike β-cells, which are found clustered within islets, K-cells are dispersed throughout the gut epithelium, averaging 13 ± 1 K-cells/1000 gut epithelial cell in humans (122). Therefore while immune attack of pancreatic β-cells is easily detected by the massive infiltration of mononuclear cells, as indicated by dense hematoxylin staining (Fig. 25) or DAPI-positive nuclei staining (Fig. 2, 21-24), immune attack of the dispersed gut K-cells would not be expected to elicit such a massive immune response to individual K-cells. In fact, no discernable differences in nuclear staining were observed between duodenal sections from transgenic and non-transgenic mice that were stained for insulin, GIP and DAPI (Fig. 21-23 vs. Fig 24) or with H&E (Fig. 25). Therefore, this study looked at two leukocyte markers to investigate whether there might be increased infiltration in the duodenum of transgenic mice.

CD45, or common leukocyte antigen, is a transmembrane protein tyrosine phosphatase that is highly expressed on the cell surface of all nucleated hematopoietic cells (165). CD45 is involved with the regulation of lymphocyte development and activation; for example, CD45 has been suggested to influence cellular responses of both T cells (166) and B cells (167, 168) by regulating their relative sensitivity threshold to external stimuli. Changes in the number of CD45-positive cells can therefore provide an indication of changes in leukocyte numbers. As expected, CD45 staining in both pancreas and duodenal sections from non-transgenic and transgenic female NOD mice was restricted to what appears to be the cell-membrane (Fig. 28 and 29). In the pancreas, CD45 staining was observed around the DAPI-positive nuclei surrounding the islets (Fig. 28 and 29, top panel), positively identifying these cells as immune cells. When the duodenal sections were quantified for the leukocytes, no difference in the number of CD45-positive cells per 100 μm villus was observed between the two genotypes (Fig. 30A). Thus it appears that insulin-producing K-cells do not elicit increased leukocyte infiltration into the gut epithelium of transgenic mice compared to their non-transgenic littermates.
However, despite a lack of change in the total number of leukocytes, the possibility of changes in the population of the different types of leukocytes required examination.

The involvement of T cells in T1DM is beyond doubt. Not only are T cells present in inflammatory lesions of the pancreas (i.e. peri-insulitis and insulitis), but immunosuppressive drugs directed against T cells have been shown to delay onset and progression of the disease (169, 170). T cells are also important in the gut immune system. In the gut, a subpopulation of T cells, known as intraepithelial lymphocytes, are situated among epithelial cells where they help defend against potential pathogens that enter the intestine and influence gut epithelial homeostasis via local production of cytokines (161, 171). In light of the critical role that T cells play in T1DM disease pathogenesis and in gut immune system functioning, this study examined the duodenum of non-transgenic and transgenic mice for changes in location and number of intraepithelial T cells. Although T cells detect antigens with the T cell receptor (TCR), information about antigen/TCR interactions are relayed intracellularly via the associated CD3 subunits (172). CD3 molecules, which are transmembrane glycoproteins, are necessary for both TCR signalling and cell surface expression and thus are commonly used as a marker of T cells (172-176). Similar to CD45 immunostaining, CD3 staining in the pancreas and duodenum of non-transgenic and transgenic mice was also restricted to the cell surface, forming a ring around DAPI-stained nuclei (Fig. 31 and 32). Intraepithelial cells were found to be mainly subnuclear in location, with only a few located supranuclear in a few sections (data not shown).

Besides villus shortening and crypt hyperplasia, increased intraepithelial cell infiltration into gut epithelium is another characteristic of many intestinal diseases including celiac disease and other food-antigen related enteropathies (162). In fact, increased intraepithelial density may exist independent of gut architectural changes (162) and is one of the earliest pathological changes observed with many pathological conditions of the small intestine, particularly celiac disease, where changes in intraepithelial density may be the only indication of gluten sensitivity (177). Therefore, differences in intraepithelial cell population serves as a good indicator for the
presence of cell-mediated mucosal immunity (162). In this study, enumeration of the intraepithelial cells present in duodenal sections from non-transgenic and transgenic female NOD mice indicated that there was no difference in intraepithelial populations between the two genotypes (Fig. 30B). It is of interest to note that the density of intraepithelial cells observed in this study (8.9 ± 1.3 and 7.9 ± 0.4 CD3-positive intraepithelial cells/100 duodenal epithelial cells for non-transgenic and transgenic mice, respectively) is fairly low compared to the previously observed density of 22.1 ± 5.1 (178). In fact, the density observed in this study more closely resembles the density of 12.0 ± 3.9 previously reported in the ileum (178). This difference may reflect a strain difference, since this study looked at NOD mice while the previous study by Tamura (178) used BALB-c mice. Overall, these results suggest that insulin-producing K-cells do not elicit increased cell-mediated immunity compared to normal K-cells that don't produce insulin and thus do not appear to be targeted by the gut immune system.

Similar to the observed absence of autoimmunity against K-cells engineered to secrete insulin, Lipes et al. (26) also found that insulin-secreting intermediate lobe pituitary cells were not destroyed by the autoimmune system of NOD mice. Using a rat proopiomelanocortin (POMC) promoter / mouse preproinsulin II (rPOMC/mINS) transgene, insulin expression was targeted to the POMC-expressing pituitary cells in NOD mice (26). Serial examination of pituitaries from rPOMC/mINS transgenic NOD mice showed no ectopic lymphocytic infiltration, even after onset of diabetes (26). Furthermore, transgenic pituitary cells transplanted under the kidney capsule of diabetic non-transgenic NOD recipients not only reversed diabetes but were also resistant to the autoimmune destructive process that completely destroyed the insulin-producing β-cells of islets that were either co-transplanted under the capsule of the contralateral kidney or mixed together and engrafted with the pituitary cells under the same kidney capsule (26). Thus even direct contact with islet-specific pathogenic lymphocytes did not result in immune-mediated destruction of the insulin-expressing pituitary cells (26). Together, the
findings of this thesis and of Lipes et al. (26) suggest that ectopic insulin expression alone does not provoke autoimmune infiltration or destruction of the engineered cells.

However, considering that the studies reported in this thesis were conducted on NOD mice, care must be taken when extrapolating the findings to humans. Since its discovery, the NOD mouse has become the favored animal model for studying the etiopathogenesis of T1DM (179). Reasons for this popularity include: 1) its fairly well-characterized genome; 2) availability of monoclonal antibodies for analyzing components of the immune system; and 3) relatively low maintenance costs (179). Many studies focused on different treatments and preventative therapies have also been conducted on the NOD mice with positive findings. In fact, based on the protective effects observed in NOD mice (180-183), clinical trials with anti-CD3 therapy have been conducted and yielded promising effects at slowing the progression of T1DM in humans (170, 181). Yet many other methods or therapies that were found to prevent, delay or slow T1DM in NOD mice have failed to show similar protective effects in humans (e.g. oral insulin therapy discussed earlier), emphasizing the limitations of this animal model of T1DM. Due to genus-specific features that distinguish murine and human diabetes, the interpretation of results generated from NOD mice are restricted. Some of the limitations of using NOD mice include: 1) differences in disease pathology such as the accumulation of T cells in pancreas as well as peripheral lymphoid organs and submandibular glands in NOD mice, which has been attributed to low levels of IL-2 and resistance of thymocytes and peripheral T cells to apoptosis (179); and 2) immunodeficiencies of NOD mice that have arisen due to inbreeding, such as impaired APC and T cell communication and impaired ability of macrophages to activate regulatory T cells (179). Therefore, while the findings of this thesis indicate that insulin-producing K-cells are not targeted by the immune system of transgenic NOD mice, the differences between T1DM in human and NOD mice and the immunodeficiencies of NOD mice must be kept in mind.

Furthermore, clinical application of targeting insulin production to gut K-cells for treatment of T1DM in humans will require the development of appropriate gene delivery techniques. Gene
delivery relies on using vectors to transfer the gene of interest into the body. Of the two main types of vectors used in gene therapy research, namely non-viral and viral vectors, viral vectors are more commonly used due to its ability to deliver genes with higher efficiency and to target a wider range of host cells (184). However, while positive clinical findings (e.g. the successful treatment of patients with severe combined immunodeficiency (SCID) (185, 186)) have demonstrated the potential of gene therapy for treating a vast array of diseases, several clinical setbacks have highlighted the risks involved with gene therapy. The recent death (the precise cause of which is still unknown at the time of thesis submission) of a patient involved in a phase I/II gene therapy clinical trial for the treatment of active inflammatory arthritis resulted in the suspension of this clinical trial by the Food and Drug Administration (FDA) (187-189). This death is the third major blow to the field of gene therapy. In 1999, an 18-year old male patient also died following high-dose adenoviral gene therapy for a rare liver disease (190). Similarly, although gene therapy successfully cured patients with SCID, it was also linked to the development of leukemia in two babies (185, 186, 191). Therefore, in light of the limitations and obstacles associated with gene therapy, additional research focused on further characterizing insulin production from modified gut K-cells and on developing safe and effective gene delivery techniques is needed before this approach (i.e. targeting gut cells for insulin replacement) can be used for treating T1DM in humans.
CONCLUSION

Until recently, despite successful targeting of insulin production to many non β-cells, a major limitation of these attempts has been the lack of meal-dependency of insulin secretion in these surrogate cells. In 2000, Cheung et al. (44) showed that the gut K-cells may be a suitable β-cell surrogate. Using a rGIP/hINS transgene, meal regulated insulin secretion from gut K-cells was achieved and was able to prevent the development of diabetes and glucose intolerance following treatment with the β-cells toxin, STZ (44). However, a potential obstacle for the development of this approach as a therapy for insulin replacement is autoimmunity. While Lipes et al. (26) showed that pituitary cells expressing insulin are not targeted and destroyed by the immune system of NOD mice, a great deal of evidence has accumulated that implicates insulin in the selective destruction of β-cells. Proinsulin, or more specifically insulin peptide B9-23, has also recently been suggested as an essential target of the immune system of NOD mice (87). Thus whether insulin production in K-cells in the setting of autoimmune diabetes might elicit an immune response to the engineered K-cells warranted careful examination. Insulin expression in gut K-cells of transgenic NOD mice containing a rGIP/hINS transgene was confirmed by the colocalization of insulin- and GIP-immunoreactivity. Considering the target specificity of antibodies and immune cells and reduced biological effectiveness (receptor binding and biological activity) of incompletely processed insulin, gut proinsulin processing was investigated. By immunostaining for processed forms of insulin and various insulin processing hormones, this study found that from the duodenum to the ileum of the small intestine, only a portion of insulin-positive gut cells contained insulin processed at the BC junction, and/or the processing enzymes CPE and PC2. Thus these preliminary quantification studies suggest that the majority of proinsulin in the gut is only partially processed. Despite the suggested processing defects, not only were female transgenic mice with insulin-producing K-cells protected from developing chemically-induced diabetes, they were also protected from developing spontaneous
autoimmune-mediated diabetes. In fact, the observed phenomenon of little or no detectable insulin in the pancreas with many insulin-positive K-cells and normoglycemia in transgenic mice suggest that in these mice, gut insulin helps to regulate blood glucose and that insulin-producing K-cells persist despite immune destruction of pancreatic \( \beta \)-cells. In light of the fact that the gut mucosa renews itself every 3-5 days, gross gut morphology and leukocyte counts were investigated to better detect possible immune attack of modified K-cells. In these studies, no detectable gross morphological difference was observed in the duodenum of transgenic mice compared to their non-transgenic littermates. More specifically, villus and crypt lengths, epithelial thickness (i.e. crypt-villus lengths) and villus to crypt ratio were similar between the two genotypes, suggesting that there was no increased gut inflammation in transgenic mice compared to non-transgenic mice. K-cells were also evenly distributed along the crypt-villus axis in the duodenum of both genotypes. In fact, the mean duodenal K-cell location of transgenic mice was significantly, albeit slightly, closer to the villus tip than that of non-transgenic mice. This finding suggests that modified K-cells are not being destroyed before reaching the villus tip. Furthermore, no differences in the number of CD45-positive (normalized to villus length) nor density of CD3-positive intraepithelial cells were observed in the duodenum of the two genotypes. Thus the presence of insulin in the gut is not associated with detectable changes in total number of leukocytes or density of intraepithelial T lymphocytes in the duodenum. The results from these current studies suggesting an absence of detectable autoimmunity against modified K-cells further supports targeting insulin production to gut K-cells as a treatment for diabetes and encourages further investigation into optimizing gene delivery techniques to the small intestine to establish long-term GIP-insulin transgene expression.
FUTURE STUDIES

The results of this thesis raise several possible future studies to more thoroughly evaluate the autoimmunogenicity of gut insulin. First, a more detailed examination of leukocytes should be performed. Instead of simply evaluating the number of CD3-positive intraepithelial cells, CD4-positive and CD8-positive T cells could be quantified. Results from transgenic and knockout mice studies and T cell (both CD4-positive and CD8-positive) transfer studies support the essential involvement of both sets of T cells in the initiation of T1DM in NOD mice (55, 192-194). NOD mice lacking major histocompatibility complex (MHC) class I molecules fail to activate CD8-positive T cells and do not develop insulitis or T1DM (194). Furthermore, the ability of CD8-positive T cells to home to islets is believed to be dependent on CD4-positive T cells (195). In contrast to the necessity of both T cell populations in the initiation of disease, CD4-positive T cells can transfer diabetes to recipient mice in the absence of CD8-positive T cells, suggesting that disease progression is facilitated by but not dependent on CD8-positive T cells (193, 196). Finally, there are two subpopulations of CD4-positive T cells, T<sub>H1</sub> and T<sub>H2</sub> cells, with differential cytokine secretion and effects on T1DM disease progression. While T<sub>H1</sub> cells, which preferentially secrete the proinflammatory cytokines interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α), contribute to β-cell destruction, T<sub>H2</sub> cells preferentially secrete the anti-inflammatory cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 and prevent disease progression (55, 192). Furthermore, a skewing towards T<sub>H1</sub> cells, as indicated by the cytokine secretion profiles of islet-infiltrating T cells, has been observed in prediabetic NOD mice (55, 65). In 4 to 6-week old female NOD mice, the ratio of IFNγ to IL-4 expression is high and is predictive of both onset of destructive insulitis and incidence of T1DM in these mice (159). Therefore, by only quantifying the overall intraepithelial T cell density, this thesis might have missed changes in the subpopulations of T cells. Therefore a closer examination of the T cell populations in the gut of transgenic NOD mice and their littermates should be conducted. More specifically,
besides quantifying the population of CD4-positive and CD8-positive T cells in the gut, the ratio of T\textsubscript{H}1 to T\textsubscript{H}2 cells should be determined by, for example, comparing the levels of the cytokines secreted by these cells to evaluate if insulin-producing K-cells evoke a change in the proportion of gut proinflammatory and immunoregulatory T cells.

Second, considering that abundant insulin-immunoreactivity was observed in some older (≥ 10-month old) transgenic NOD mice, the tolerogenic effect of gut insulin in the pancreas should be investigated. As discussed earlier, oral insulin has been shown to induce tolerance and protect NOD mice from developing diabetes (111, 112). Therefore, insulin production in the gut may induce a similar tolerogenic effect, resulting in the protection of β-cells from autoimmune-mediated destruction, and thereby contributing to the decreased incidence of diabetes observed in transgenic NOD mice compared to their non-transgenic littermates. In order to examine this possibility, the pancreases of non-transgenic and transgenic NOD mice should be systematically examined. More specifically, the degree of lymphocyte infiltration and insulin-immunoreactivity should be scored (e.g. 1 = no lymphocyte infiltration; 2 = peri-insulitis; 3 = insulitis; and 4 = no lymphocyte infiltration and no insulin-immunoreactivity) on multiple pancreatic sections taken from throughout the pancreas (e.g. every 4\textsuperscript{th} tissue section of each pancreas sectioned into 3-5 μm thick sections). In addition, since the surface expression of CD25, the receptor for IL-2, on T cells is upregulated in response to T cell activation (197, 198), pancreas sections should also be immunostained for CD25 to assess T cell activation and proliferation. A closer examination of the T cell subpopulations and cytokine expression in the pancreas should also be conducted to evaluate the ratio of proinflammatory (T\textsubscript{H}1) to immunoregulatory (T\textsubscript{H}2) T cells and thus presence of autoimmunity or tolerance induction.

Third, since antigen signal is an important factor for triggering an immune response, any potential immunogenic signals from the dispersed K-cells in the gut epithelium may be too weak to generate a response. Also, perhaps life-long gut insulin expression results in tolerance to gut insulin. Since the ultimate goal of targeting insulin replacement to the gut K-cells is to induce
insulin-expression in the gut of patients with T1DM, which thus will likely occur post-natal after
the immune system has been fully developed, distinguishing the potential autoimmunogenicity
from potential tolerogenicity of gut insulin is important. Therefore, to determine whether insulin-
producing K-cells are immunogenic, isolation and then transplantation of K-cells under the
kidney capsule of diabetic NOD mice could be performed. Furthermore, it is possible that
activation of insulin-specific lymphocytes is dependent on some cell injury event that attracts
sufficient quantities of antigen-presenting cells to activate relevant lymphocytes (Lipes 1996).

To address this possibility, isolated K-cells could be mixed and implanted with islets under the
same kidney capsule of diabetic NOD mice, where elevated glucose levels (and perhaps
elevated lipid levels) results in β-cell damage (i.e. gluco- and lipotoxicity (199, 200)) and
subsequent immune activation. If insulin-producing K-cells are immunogenic but simply lack
sufficient numbers of APCs to trigger lymphocytic infiltration, then placing them with islets
should result in the parallel destruction of modified K-cells and β-cells. In contrast, if insulin-
producing K-cells are not immunogenic and are resistant to immune-mediated destruction, then
these cells should persist despite lymphocytic infiltration and destruction of insulin-producing β-
cells of the transplanted islets. However, considering that there are currently no known K-cell-
specific markers that can be used for isolation purposes, transgenic mice with fluorescently
labeled K-cells (e.g. transgenic mice expressing a GIP promoter / red fluorescent protein (RFP)
transgene) could be bred with the transgenic NOD mice used in this thesis to generate mice
containing K-cells that express both insulin and red fluorescent protein. Alternatively, transgenic
mice could be generated with a new transgene construct expressing both insulin and RFP or
green fluorescent protein (GFP) off of the GIP promoter. Either way, K-cells could then be
isolated by fluorescence-activated cell sorting (FACS) for the fluorescent protein following
mechanical and enzymatic digestion of the gut epithelium.

Fourth, although no difference in K-cell survival/destruction was observed between non-
transgenic and transgenic NOD mice, a comparison of gut samples to a positive control of K-cell
destruction would strengthen the finding. By using cell-specific promoters to regulate expression of viral proteins, virus-induced autoimmune destruction of specific cells has been demonstrated. For example, the RIP-LCMV-gp mouse, a mouse model of virus-induced T1DM, expresses a lymphocytic choriomeningitis virus (LCMV)-derived glycoprotein (gp) under the control of the rat insulin promoter (RIP) (201-203). The expression of the transgene is restricted to the pancreatic β-cells and does not naturally lead to islet infiltration or destruction. However, when these transgenic mice are infected with LCMV, self (viral gp)-tolerance is broken and T1DM develops rapidly following virus-specific T cell activation and cytokine-mediated destruction of β-cells (201-204). Although this particular model targeted the β-cells, the same concept could be applied to the gut K-cells. Thus virus-induced autoimmune-mediated destruction of K-cells could be achieved by generating transgenic mice expressing a GIP-LCMV-gp transgene, where LCMV-gp is expressed under the control of the GIP promoter. Duodenal tissues from LCMV-treated GIP-LCMV-gp transgenic mice could then serve as a positive control of autoimmune-mediated K-cell destruction.

Fifth, further studies should also be conducted to better characterize transgene expression and confirm tissue specificity of this transgene. Under the control of the GIP promoter, transgene expression should be limited to tissues with the necessary transcription factors to bind and activate the promoter. The tissue distribution of transgene expression could be determined by performing RT-PCR on RNA samples extracted from various tissues of the body (including brain, bladder, fat, gut (duodenum, jejunum, ileum, colon), heart, hypothalamus, kidney, liver, lung, muscle, pancreas, spleen, thymus and tongue) using mouse insulin primers. ISH for preproinsulin could then be performed to confirm RT-PCR results and show cellular localization of insulin gene expression. GIP-promoter activity could also be investigated using transgenic mice expressing GFP or RFP under the control of the GIP promoter. In these mice, the presence of fluorescence would reveal the tissues in which the GIP promoter is active. Based on the RT-PCR, ISH and promoter activity findings, transgene protein expression could
then be confirmed by the colocalization of GIP and insulin-immunoreactivity in double-stained tissue sections.

Lastly, since single amino acid changes can result in alterations of protein structures and thus of receptor binding sites and potential (auto)epitopes, insulin processing in the K-cells should be further examined to determine the predominant form of insulin/proinsulin intermediate(s) present in K-cells. The processing enzymes present in K-cells should also be confirmed with larger sample sizes. To better evaluate proinsulin processing at the AC junction, an antibody that recognizes the processed PC2 site, either recognizing the C terminus of C peptide or the N terminus of the A chain, should be generated. In addition, besides using immunohistochemistry to evaluate proinsulin processing, other techniques should be employed to determine the nature of the insulin produced from modified gut K-cells. Major limitations of immunohistochemistry include its dependence on the availability and specificity of antibodies and the subjectivity involved in the interpretation of observed fluorescence (i.e. in what is considered a positive cell). As such, the presence or absence of proteins may be misportrayed by immunohistochemistry. For example, the MAb1 antibody used in this study shows reduced cross-reactivity with mouse insulin due to amino acid differences between human and mouse insulin at amino acid B29 (methionine instead of lysine in mouse insulin II) and B30 (serine instead of threonine in mouse insulin I and II) (205). According to the manufacturer's product sheet, MAb1 shows only 22% cross reactivity with mouse insulin compared to human insulin. As such, the MAb1 quantifications performed in this thesis likely underestimated the actual population of modified gut K-cells producing insulin processed at the BC junction. In contrast, other analytical techniques, such as mass spectrometry and reverse phase high performance liquid chromatography (RP-HPLC), allow for unambiguous separation and quantification of proinsulin, insulin and its various intermediates, the latter of which has been previously used for such a purpose (127, 130, 131, 206). Therefore, in light of the limitations associated with immunohistochemistry, mass spectrometry and/or RP-HPLC should also be used to further (and
perhaps more accurately) characterize the insulin produced from the gut K-cells. Based on these results, the rGIP/miNS construct can then be modified to compensate for the processing enzymes that are lacking in the majority of K-cells so that the insulin produced from K-cells expressing this modified transgene will be the least immunogenic (i.e. least likely to evoke an immune response) and most biologically active.
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ANIMAL CARE CERTIFICATE

Application Number: A05-1832

Investigator or Course Director: Timothy J. Kieffer

Department: Physiology (to be delete)

Animals:

- Mice NOD/GIPIns 200
- Mice C57BL/6J, timed-pregnant 50
- Mice C57BL/6Cr; with duodenal catheters 50
- Mice NOD/LtJ, timed-pregnant 50
- Mice FVB/N/Switch/GIPIns 200
- Mice CD1 100
- Mice Factor IX knockout; B6.129P2-F9^mlDws/J 50
- Rats Sprague-Dawley 100
- Mice FVB/N 200
- Mice NOD/LTJ 100
- Mice C57BL/6 1000

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- Funding Agency: Engene, Inc
  Funding Title: CRA: Production of therapeutic proteins from gut cells.
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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

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