Unravelling beta cell destruction in type 1 diabetes

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CHAPTER 1

General introduction

1. TYPE 1 DIABETES

Type 1 diabetes (T1D) is a chronic disease characterized by the immune-mediated destruction of the pancreatic beta cells, resulting in insufficient insulin production and impaired glucose homeostasis. The symptoms include polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and unexplained weight loss. Over 40 million people worldwide suffer from T1D and yearly 80,000 new diagnoses are made. Without medical intervention the consequences of T1D are fatal. More than a century ago, the Nobel Prize-winning discovery of Banting and Best paved the way for modern day insulin therapy. Once diagnosed, patients require lifelong insulin therapy to compensate for lost beta cell function. Yet, artificial regulation of blood glucose homeostasis is not accurate and the majority of patients have difficulties to keep their blood glucose levels within the recommended range. The fluctuations between hyperglycemia (high blood glucose) to hypoglycemia (low blood glucose) can result in acute life threating conditions such as diabetic ketoacidosis, nonketotic hyperosmolar syndrome, seizures, and coma[1]. T1D is also associated with diverse long-term complications such as diabetic retinopathy, nephropathy, neuropathy, and cardiovascular diseases[2].

Currently there is no cure for T1D but methods of beta cell replacement are rapidly developing[3]. Pancreas transplantation offers improved endogenous glycemic control of T1D patients[4]. However, the invasiveness of the procedure and the need for continuous immunosuppressive drugs treatment to prevent graft versus host disease and the irreversible effect on kidney function toxicity does not outweigh the benefits[5]. In 2000, the Edmonton protocol for islet transplantation offered a new less-invasive strategy to restore endogenous beta cell function by islet cell transplantation. While follow-up studies showed promising results with improved life quality by improved glycemic control and less hypoglycemic episodes, the majority of transplanted patients did not preserve long-term insulin independence[6-8]. Though improvement in the islet isolation procedure, site of transplantation, reduction of toxic immunosuppression, and immune-protection devices may improve clinical outcome, replacement therapies (whole pancreas or purified islets) are facing shortage in organ donation. In order to overcome the lack of organ donors alternative sources of beta cells (i.e. embryonic stem cells or induced pluripotent cells) are examined[9, 10]. Also, reactivation and preservation of persistent beta

cells in T1D patients by specific or non-specific immune intervention therapy are currently under investigation[11-13].

2. HUMAN PANCREATIC ISLET PHYSIOLOGY

The human pancreas is a mixed gland that consists of an exocrine and endocrine compartment, exerting a function in the digestive system and endocrine system, respectively. The endocrine compartment, which makes up 1-2% of the total organ weight, consists of clusters of endocrine cells scattered through the exocrine pancreas collectively called the islets of Langerhans. The islets of Langerhans are assembled of different endocrine cells: the glucagon-producing alpha cells (circa 30%), the insulin-producing beta cells (circa 60%), the somatostatin-producing delta cells (circa 5%), the pancreatic polypeptide-producing gamma cells (circa 3-5%), and the ghrelin-producing epsilon cells (<1%). Together, these endocrine cells orchestrate blood glucose homeostasis by tightly regulated hormone secretion, maintaining blood glucose levels in a narrow physiological range[14]. This is mainly acquired by the balanced secretion of insulin and glucagon. In fasting conditions, when blood glucose levels tend to decrease, glucagon is released from the alpha cells. Glucagon promotes hepatic glycogenolysis increasing endogenous blood glucose levels[15]. In contrast, elevated blood glucose levels after food intake stimulate insulin secretion from the beta cells. Insulin lowers blood glucose levels by stimulating glucose uptake from the blood and storage by cells in the form of glycogen[16]. Somatostatin, ghrelin and pancreatic polypeptide indirectly contribute by modulating insulin and glucagon secretion and promoting insulin sensitivity[17-19].

The unique islet cytoarchitecture, favoring heterotypic associations, likely contribute to cell-cell communication and fine-tuning of coordinated hormone secretion[20-22]. Although the endocrine cells within the islet of Langerhans each occupy different roles in nutrient metabolism, they all share a common origin in their development[23]. A complex network of transcription factors during early pancreas development drives lineage commitment and endocrine cell diversity[24, 25]. Elaborate examination of hormone expression in human pancreatic sections from normal cadavers ranging from pre-neonatal to elderly individuals indicates that the beta cell mass is established before the age of 5

years, after which endocrine cell proliferation was rarely observed[26]. In matured endocrine cells the differentiated state is maintained by reinforcement of cell-specific gene regulatory networks and repression of other transcriptional programs[27-30].

Within the islets of Langerhans, the beta cells are micro-factories that produce, store, and release insulin[31]. Insulin is synthesized as a 110-amino acid precursor molecule, preproinsulin, consisting of a signal peptide (SP), B-chain, C-peptide and A-chain. As soon as the N-terminal signal peptide emerges from the ribosome, it is bound by signal recognition particle (SRP) and directed to translocon Sec61. The signal peptide is removed and the remaining proinsulin molecule is cotranslationally translocated to the endoplasmic reticulum (ER)[32]. Here proinsulin is folded in the correct conformation and three disulfide bonds are formed[33]. Proinsulin is subsequently transported from the ER to the Golgi apparatus where it enters immature secretary vesicles and is finally processed to bioactive insulin and its by-product C-peptide by prohormone convertases[34, 35]. In order to adequately adapt to acute changes in extracellular glucose levels, beta cells are highly sensitive to fluctuations in glucose levels. Beta cells equalize to circulating blood glucose levels by the uptake of glucose via glucose transporter 1 (GLUT1)[36]. Internalized glucose is converted to pyruvate that results in the generation of ATP. The increasing ATP/ADP ratio results in the closure of ATP-sensitive K^+-channels, membrane depolarization and opening of voltage-dependent Ca^{2+}-channels. The influx of calcium into the cell triggers the exocytosis of stored secretory granules as well as promote insulin gene transcription to replenish insulin granules and maintain glucose-stimulated insulin secretion (figure 1). Calpain-1, a Ca^{2+}-activated protease, cleaves granule transmembrane protein islet cell autoantigen 512 (ICA512 or IA-2). The cytosolic fragment of ICA512 is targeted to the nucleus where it upregulates transcription of secretory granule genes and insulin[37-39]. Insulin synthesis rates up to 1,000,000 molecules per minutes can be achieved which comprises about 50% of the total beta cell proteome[40].

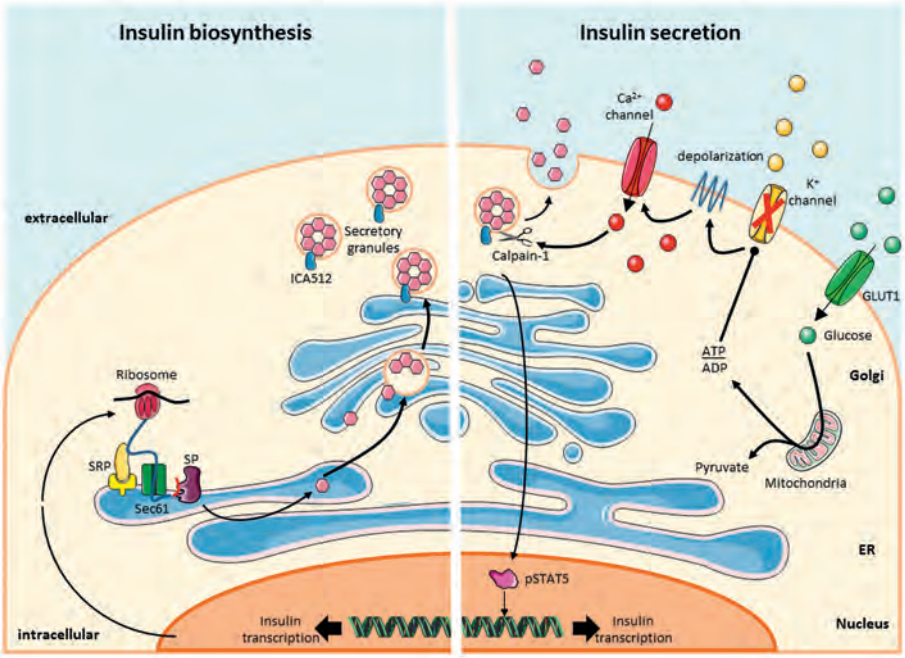

Figure 1: Insulin synthesis, storage, and secretion from a pancreatic beta cell. Insulin biosynthesis, storage in secretory vesicles (left panel), and insulin secretion (right panel) are activated by the influx of Ca2+ after membrane depolarization caused by the influx and ATP-generating metabolism of glucose. Figure was produced using Servier Medical Art www.servier.com.

3. IMMUNOPATHOLOGY OF TYPE 1 DIABETES

Genome-wide association studies have established a strong genetic risk to T1D and more than 60 candidate susceptibility loci have been identified[41, 42]. The highest risk is ascribed to immune-associated genes (50%), especially HLA class II alleles. The DR4/DQ8 or DR3/DQ2 haplotypes account for 80-90% of the patients[43, 44]. Albeit to a lesser extent, HLA class I was also shown to contribute to T1D risk[45]. In addition, many non-HLA genes have been found to associate with T1D although at lower odd ratios. These include: variation of a variable number of tandem repeats (VNTR) upstream of the insulin gene[46], autoimmune regulator (AIRE)[47], cytotoxic T-lymphocyte antigen (CTLA-4)[48, 49], lymphoid-specific protein tyrosine phosphatase 22 (PTPN22)[50], and IL-2 receptor alpha (IL2RA or CD25)[51]. These risk factors might exert their effects by poor insulin representation in the thymus and defective peripheral immune regulation by inhibited T cell repression. Besides immune regulation, the associated loci might

participate to T1D pathogenesis via different mechanisms, such as inadequate beta cell function, apoptosis signaling and interferon (IFN) signaling [52] .

The earliest indicators of the disease are accumulation of autoantibodies against beta cell antigens such as insulin (IAA), Glutamic acid decarboxylase (GAD), Islet antigen-2 (IA-2), and Zinc transporter 8 (ZnT8). Although the presence of multiple autoantibodies can be considered as biomarker for disease progression and largely increases the risk of developing T1D[53-55], their contribution to the cascade of events leading to beta cell destruction and disease progression remains unclear[56].

The dynamics of the islet microenvironment and beta cell destruction is difficult to investigate in humans as non-invasive *in vivo* imaging techniques of pancreatic islets are challenging[57] and the use of animal models to study T1D development remains controversial[58, 59]. The biobank Network for Pancreatic Organ Donors with diabetes (nPOD) provides a wide collection of human pancreatic donor material for research[60]. Analysis of pancreatic sections of T1D patients demonstrated the infiltration of a diverse collection of immune cells of both the innate and adaptive immune system in the islet micro-environment, including macrophages, natural killer cells, dendritic cells (DCs), B-cells, $CD4^+$ T cells, regulatory T cells (T_{regs}), and primarily $CD8^+$ T cells (figure 2)[61, 62].

a. Role of central tolerance

The immune system is an assembly of delicate physiological mechanisms to protect the host from pathogens. T cells are able to distinguish healthy cells from infected cells by the recognition of peptides presented on the cellular surface by the human leukocyte antigen (HLA) complex.

Peptide antigens are presented by two classes of HLA complexes, HLA class I and class II, and two specialized types of T cells respond[63]. HLA class II expression is limited to professional antigen-presenting cells (APC), DCs, B-cells and macrophages, and HLA molecules mainly present peptides derived from degraded extracellular material taken up via phagocytosis. Upon recognition by $CD4^+$ T cells, the $CD4^+$ T cells help the immune response by stimulating antibody secretion by B cells and support $CD8^+$ T cell activation. HLA class I is assembled with peptides derived by the proteasomal degradation of intracellular protein. $CD8^+$ T cells recognize peptides presented by HLA class I on the surface of all nucleated cells. Activation of $CD8^+$ T cells upon recognition of their target results in destruction of the antigen-presenting cell.

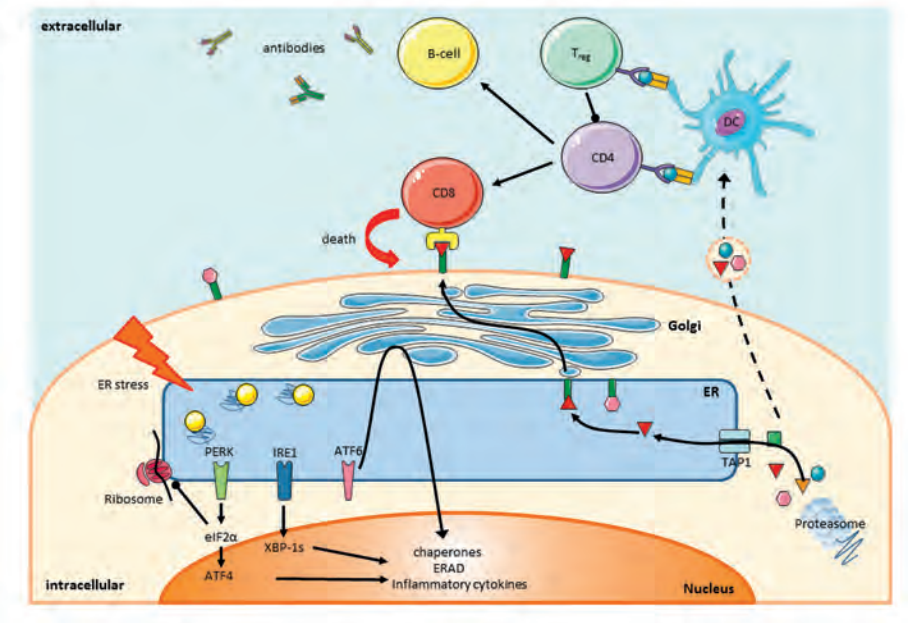

Figure 2: Antigen processing, presentation and immunology. Degraded intracellular proteins are processed and loaded in HLA class I molecule and presented at the cell surface. Antigens can also be taken up and processed by DCs via phagocytosis and presented in HLA class II. DCs regulate peripheral tolerance. If this fails recognition of epitope at the cell surface by CD8 can induce cell death. In case of T1D pathology, these epitopes are most likely from beta cell-specific proteins. The unfolded protein response activated by endoplasmic reticulum stress and the dissociation of BiP from PERK, IRE1 and ATF6 lead to inhibition of translation, increased folding, and degradation of proteins. UPR-induced inflammatory cytokine production can activate the immune system and affect peripheral tolerance mechanisms. Figure was produced using Servier Medical Art www.servier.com.

T cell recognition of foreign peptides is essential for immune defense against pathogens, however, recognition of self-peptides can cause autoimmunity. Lymphocyte receptors are highly variable as the result of gene rearrangements, allowing them to recognize an almost unlimited repertoire of antigens. In order to discriminate pathogenic peptides from self-peptides, lymphocytes are subjected to a delicate selection process to generate central tolerance[64]. This two-step selection process in the thymus consist of positive selection of T cells with a T cell receptor (TCR) that recognize HLA and, subsequently, negative selection of TCRs reactive to self-peptides. This ensures only self-tolerant naive lymphocytes enter the circulation.

b. The specific destruction of beta cells

The immune infiltrate in islets of T1D patients suggests substantial crosstalk between innate and adaptive immune cells, adding to the complexity of the disease[65]. However, the specific and selective destruction of the beta cells in the islet of Langerhans dictates a pivotal role for $CD8^+$ T cells that target endogenous epitopes derived from beta cell-specific proteins presented by HLA class I molecules on the beta cells. In addition, $CD8^+$ T cells are the predominant component of the immune infiltrate found in T1D patients and increasing numbers of cytotoxic T cells is associated with increased beta cell exhaustion[61, 62]. Depletion of $CD8^+$ T cells in mice was found to reduce diabetic penetrance and adoptive transfer of $CD8^+$ T cells can induce diabetes[66, 67]. Knockout of MHC class I (HLA class I equivalent) prevents development of diabetes[68]. Also, studies in human pancreatic islets demonstrate the relevance of $CD8^+$ T cells. Autoreactive beta cell specific $CD8^+$ T cells are expanded in peripheral blood of patients and are capable of killing beta cells *in vitro*[69].

Insulin is considered a key autoantigen in the development of T1D as proinsulin and insulin are common targets of autoantibodies, $CD4^+$ and $CD8^+$ T cells among (pre-)T1D patients[70, 71]. Characterization of $CD8^+$ T cell epitopes indicate the leader sequence[69, 72], the B-chain[73, 74] as well as the C-peptide[75] are implicated in autoimmunity. Beside insulin, GAD65, islet-specific glucose-6-phosphatase catalytic subunit (IGRP)[76, 77], chromogranin A[78], islet amyloid polypeptide (IAPP)[77], ZnT8[79, 80] and tyrosine phosphatase-like insulinoma antigen 2 (IA-2)[81] were identified as targets of autoreactive T cells.

Defects in T cell selection might result in the circulation of autoreactive T cells with as consequence the inappropriate T cell-mediated targeting and destruction of healthy peptide-presenting cells. Insulin expression levels in the thymus is important in the generation of self-tolerance. Expression of insulin in the thymus is controlled by the variable number of tandem repeats (VNTR) in the insulin promotor and autoimmune regulator (AIRE)[82]. Genetic polymorphisms in the VNTR are associated with disease susceptibility, in which short VNTR alleles (class I) predispose to T1D and longer VNTR alleles (class III) protect[46, 83]. Low thymic insulin levels resulted in more circulating insulin-reactive T cells, likely caused by a failure of negative selection of insulin-specific T cells[84]. Another important factor in T cell education is peptide representation by HLA. An inverse correlation between binding affinity of beta cell peptides to HLA class I and corresponding $CD8^+$ T cell responses has been reported[85].

This indicates that low affinity peptides are less represented during thymic selection. These findings led to the identification of several PPI epitopes with very low HLA class I binding affinity[86, 87].

c. Role of peripheral tolerance

Even though the role of $CD4^+$ and $CD8^+$ T cells in T1D pathology is clearly demonstrated, the circulation of these autoreactive T cells is also found in peripheral blood of healthy individuals[88-90]. This indicates central tolerance is not absolute and likely peripheral tolerance mechanism are involved to prevent activation of these T cells. APCs (B-cells, DCs, and macrophages) and a specialized subset of regulatory T cells (T_{regs}) are considered important modulators of T cell activity. The interaction between APC and T_{regs} induces a tolerogenic environment inhibiting peripheral autoreactive $CD4^+$ and $CD8^+$ T cells. Multiple lines of evidence indicate that T1D patients are unable to maintain peripheral tolerance due to altered APC and T_{reg} function[91, 92]. This potentially contributes in loss of immunoregulatory function, stimulating a pro-inflammatory microenvironment and subsequent T cell-mediated beta cell destruction.

4. ISLET MICROENVIRONMENT AND LOSS OF TOLERANCE

The incidence of T1D is increasing faster than can be explained by a shift in genetic susceptibility[93, 94]. Moreover, only a small proportion of HLA susceptible individuals actually develop T1D and the concordance of monozygotic twins with T1D is relatively low, suggesting environmental factors play an important role in disease onset[95, 96]. Several environmental factors have been associated with the development of T1D. Among them, the gut microbiome seems to be an important modulator of beta cell autoimmunity[97]. Enterovirus infection of human pancreatic islets in particular have been shown to induce secretion of cytokines and chemokines by endocrine cells and contribute to the inflammatory milue[98]. Post-transplant diabetes mellitus (PTDM) is a common complication after kidney transplantation, caused by the immunosuppressive drug tacrolimus that affects beta cell function[99]. These environmental factors are likely to increase endoplasmic reticulum (ER) stress which contributes to the induction of an inflammatory response as danger signal for the immune system[100].

ER stress leads to accumulation of unfolded or misfolded proteins in the ER lumen, which leads to activation of the unfolded protein response (UPR)[101]. In homeostatic conditions, the three ER stress sensors protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), are kept in an inactivated state by binding of ER chaperone BiP to the luminal domains of the sensors[102]. Upon accumulation of misfolded proteins, BiP dissociates from the receptors and binds to the exposed hydrophobic domains of the circulating misfolded proteins, thereby allowing self-activation of the receptors. ATF6 is translocated to the Golgi apparatus where protease digestion releases the cytosolic domain[103]. Once released, this domain moves into the nucleus where it acts as transcription factor. Both IRE1α and PERK are activated through auto-transphosphorylation. Activated IRE1α induce the unconventional cytoplasmic splicing of X-box binding protein 1 (XBP1), thereby removing a 26-nucleotide intronic region and activating XBP1 as transcription factor, XBP1 spliced. These transcription factors induce the transcription of UPR target genes that include several chaperones and genes associated with ER-associated protein degradation (ERAD). Activated PERK leads to attenuated protein synthesis by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α)[104]. The parallel signaling cascades ultimately lead to a reduction of the ER load and enhanced ER capacity by inhibiting of translation, increased degradation of misfolded proteins and increased protein folding.

When ER stress cannot be resolved, this will eventually lead to the activation of apoptotic programs[105]. However, even transient ER stress has significant consequences for the cell. ER stress can be an important indicator of pathogen invasion and cell dysfunction and therefore emits a danger signal to the immune system[106, 107]. Activation of the UPR is inevitably connected with the production of inflammatory cytokines and, in combination with hyperexpression of HLA class I[108], optimizes immune surveillance. This is achieved by modulation of nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) by the UPR signaling cascade and the subsequent transcription of proinflammatory cytokines IFN-β, IL-1β, TNF-α, and IL-6[109-111]. Beta cell autoimmunity will enhance this proinflammatory environment by secretion of IL-1β, TNFα, and IFNγ by immune cells[112, 113].

As earlier discussed, the ER is of crucial importance in the biosynthesis, maturation and secretion of insulin. Glucose directly stimulates exocytosis

of stored insulin granules from the beta cell. In addition, to replenish the insulin storage and maintain glucose-responsive insulin secretion, insulin transcription and translation are induced. Perturbation of ER homeostasis can have detrimental consequences for the beta cells[114]. Secondary environmental triggers could be the final blow, tipping the scale and distort the natural ER balance. The implication of ER stress in disease pathogenesis is supported by the finding of increased ER stress markers in the islets of Langerhans of T1D patients[115]. Research indicated that ER stress precedes the onset of T1D and supposedly serves as a trigger for β-cell dysfunction and autoimmunity[114, 116]. Moreover, insulitic islets showed overexpression of interferon-stimulated genes[117]. The inflammatory environment likely contributes to the induction and amplification of beta cell dysfunction and death[118].

Inflammation and ER stress lead to profound changes in beta cells from the conversion of the genetic information into peptides loaded on HLA molecules[119]. Transcriptomic analyses performed on human islets maintained in presence or absence of proinflammatory cytokines have revealed a clearly different gene expression profile and a high proportion of RNA alternative splicing events (over 3000 genes undergo alternative splicing in stress condition)[120]. The increased cytosolic Ca^{2+} concentrations as consequence of ER stress, impact the activity of post-translational modification enzymes i.e. tissue transglutaminase 2 (Tgase2) and peptidylarginine deiminase 2 (PAD2) that catalyzes deamidation or citrullination respectively[121, 122]. These modifications contribute to the formation of non-conventional polypeptides and immunopeptides. In case these modified proteins are not generated in adequate amounts during thymocyte maturation, T cells reactive towards this class of neo-self-epitopes are not eliminated during negative selection and are part of the matured circulating T cell repertoire[123]. Therefore tissue-specific neoepitopes provide a possible explanation for the loss of self-tolerance and rather support beta cell dysfunction as cause of disease pathogenesis, besides failure of mechanisms that induce self-tolerance.

5. AIMS AND OUTLINE OF THIS THESIS

In this thesis the interplay between the islet microenvironment and the immunogenicity of human beta cells in type 1 diabetes pathology have been

investigated. *Chapter 2* demonstrates a direct link between ER stress, the increased visibility of beta cells to the immune system, and demonstrates the importance of the endoplasmic reticulum sensors in shaping antigenic peptide presentation to CTLs. *Chapter 3* evaluates similarities between autoimmunity and an efficient anti-tumor response and proposes that beta cell autoimmunity is a well-intended immune response to dysfunctional beta cells. Furthermore the participation of the microenvironment on the generation of neoepitopes is discussed. The participation of an insulin-defective ribosomal product in T1D pathology is described in *Chapter 4*. *Chapter 5* describes the characterization of a novel luciferase reporter to evaluate the ER stress status in human beta cells. *Chapter 6* reveals the presence of a new insulin isoform in somatostatin-producing cells. The significance of all these findings in T1D research is discussed in *Chapter 7*.

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