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Unravelling beta cell destruction in type 1 diabetes

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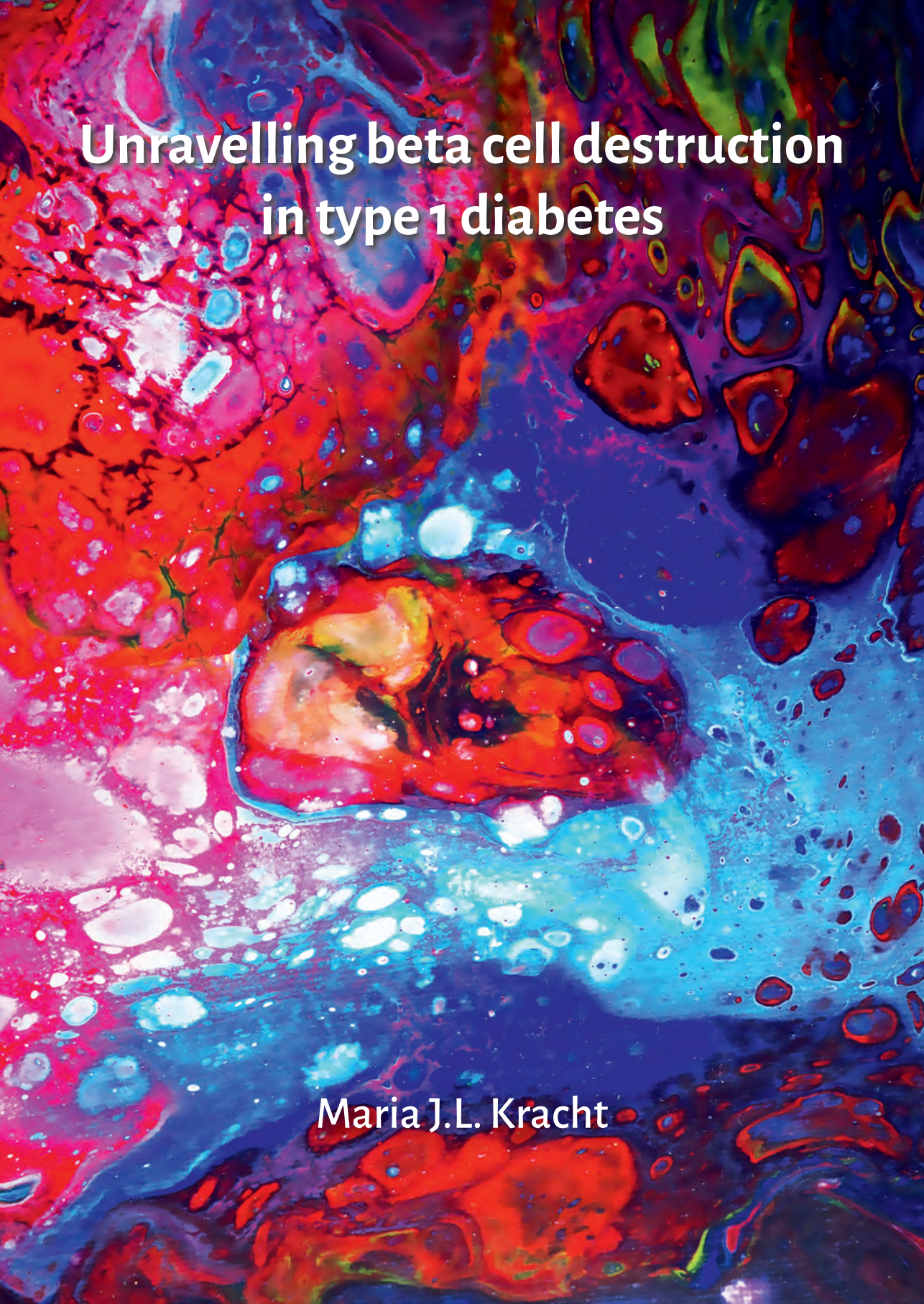

Maria J.L. Kracht

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Unravelling beta cell destruction in type 1 diabetes

PROEFSCHRIFT

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Zo is het en niet anders!

Jacoba Kracht-van der Windt
(22 september 1923 – 21 maart 2000)

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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].

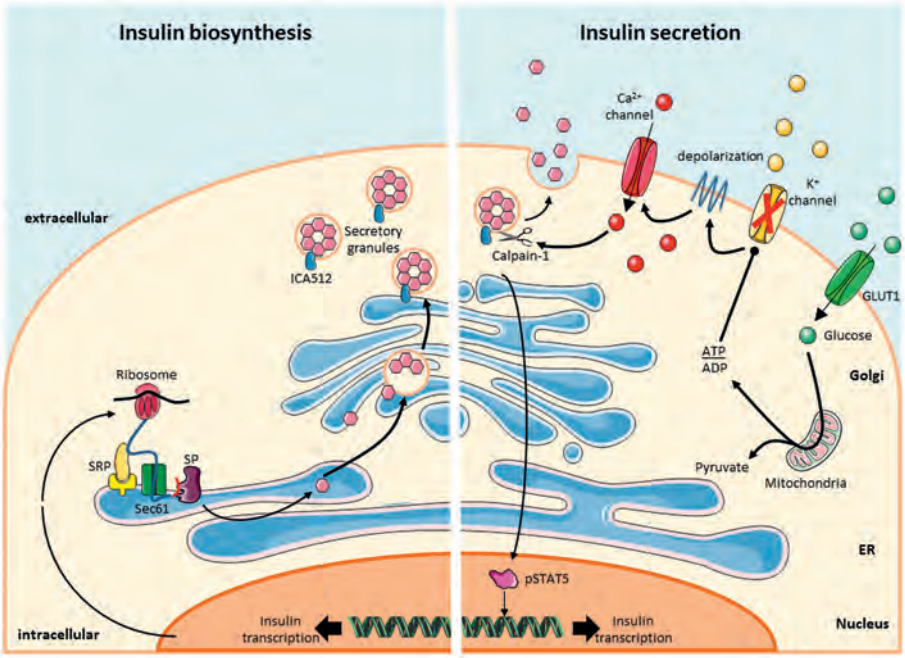

Figure 1: Insulin synthesis, storage, and secretion from a pancreatic beta cell. Insulin biosynthesis, storage in secretory vesicles (left panel), and insulin secretion (right panel) are activated by the influx of Ca2+ after membrane depolarization caused by the influx and ATP-generating metabolism of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

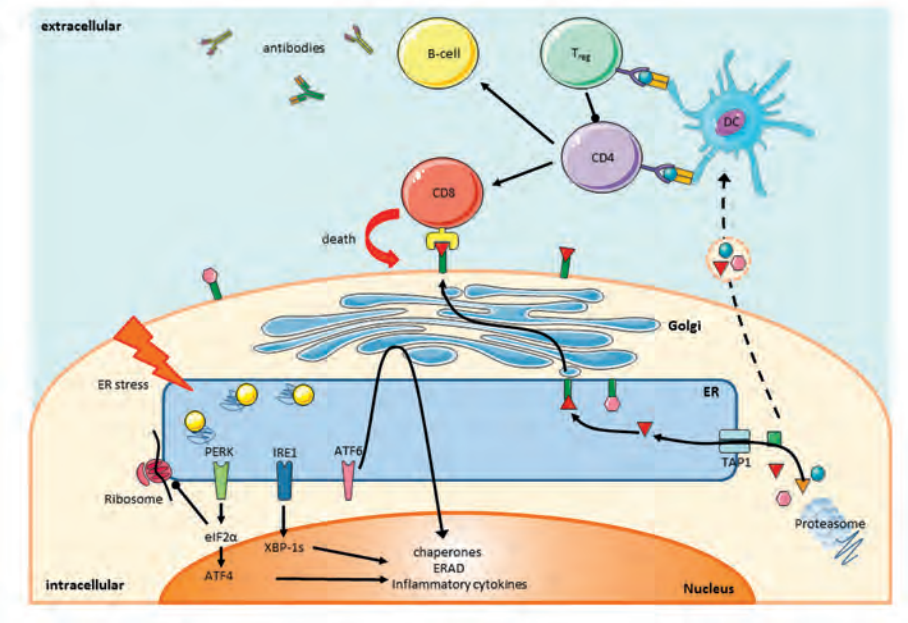

Figure 2: Antigen processing, presentation and immunology. Degraded intracellular proteins are processed and loaded in HLA class I molecule and presented at the cell surface. Antigens can also be taken up and processed by DCs via phagocytosis and presented in HLA class II. DCs regulate peripheral tolerance. 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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CHAPTER 2

Preproinsulin autoantigen processing by ER aminopeptidase 1 is upregulated during ER stress via IRE1α-mediated miR-17 degradation

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ABSTRACT

The signal peptide of the preproinsulin is a major source for HLA class I autoantigen epitopes implicated in CTL-mediated beta cell destruction in Type 1 Diabetes (T1D). The combined action of signal peptide peptidase and endoplasmic reticulum aminopeptidase 1 (ERAP1) participates to the processing of the signal peptide and to the expression of this epitope at the beta cell surface. The regulatory mechanisms controlling signal peptide trimming and the contribution of the T1D inflammatory milieu on these mechanisms are unknown. We studied the role of inflammation and ER stress on ERAP1 gene expression. Inflammation and ER stress induction in human beta cells lead to increased expression of ERAP1 and to the decreased amounts of miR-17-5p. These results confirm the importance of ER stress in the increased visibility of beta cells to the immune system and position the IRE1α/miR-17 pathway as a central component in beta cell destruction processes. This pathway therefore forms a potential target for the treatment of autoimmune T1D in recent onset patients.

INTRODUCTION

Type 1 diabetes (T1D) results from selective and progressive destruction of insulin-producing cells by autoreactive $CD8^+$ T cells[1,2]. Immunohistochemistry of insulitic pancreases obtained through the Network for Pancreatic Organ Donors with Diabetes program (nPOD)[3] have shown a massive infiltration of immune cells and an increased expression of HLA class I and markers characteristic of a cellular stress response related to endoplasmic reticulum (ER) stress, referred to as the Unfolded Protein Response (UPR)[4-6]. Altogether these results suggest a correlation between ER stress and the increased visibility of beta cells to the immune system[7]. While several proteins have been identified as potential autoreactive T cell targets, evidence from studies in mice and men suggests that insulin itself could be a main autoantigen targeted by infiltrating $CD8^+$ T cells in T1D[8-10]. The posttranslational processing pathway that generates insulin from its precursor molecule preproinsulin (PPI) is well established: the signal peptidase Sec11 cleaves off the signal peptide cotranslationally upon translocation of the protein into the ER via the translocon Sec61[11]. After proinsulin (PI) folding and the formation of three disulfide bonds, proinsulin is transported via the Golgi system into immature secretory vesicles where mature insulin is generated by the action of the prohormone convertases PC1/2. The central region of PI, the C-peptide, is cleaved out and the B- and A-chain located in amino- and carboxy-terminus, respectively remain together by the previously formed disulfide bonds yielding bioactive insulin[11-14]. Accumulating new data highlight the importance of the PPI signal peptide (SP), of 24 amino acids long, as source of insulin derived class I epitopes[15]. Peptide elution experiments performed on HLA-A2 surrogate beta cells led to the identification of prominent HLA class I binders located in the SP domain[16] and $PPI_{(SP15-24)}$-directed CTLs were found to be highly prevalent in recent onset T1D patients[17]. Recently, using cell free translocation assays and CrispR/Cas technology elegantly demonstrated the importance of the combined action of the signal peptide peptidase and ER aminopeptidase 1 (ERAP1) in the trimming of the PPI SP and in the generation of the $PPI_{(SP15-24)}$ epitope[18]. While the epitope length match with the structural characteristic of ERAP1 and the model described previously[19,20], the link between T1D pathophysiology and the immunoreactivity against $PPI_{(SP15-24)}$ remains unclear. Here, we investigated the effect of inflammation and ER stress on ERAP1 gene expression in human

beta cells and show that proinflammatory cytokines as well as chemically induced ER stress modulate ERAP1 expression through induction of miR17-5p degradation. These data establish a direct link between ER stress, and beta cell immunogenicity and demonstrate the importance of the ER sensors in shaping antigenic peptide presentation to CTLs.

RESULTS

In T1D patients, surface presentation of autoantigens in HLA class I and their recognition by $CD8^+$ T cells results in the induction of apoptotic cell-death in β-cells. To quantify CTL-induced cell death, we used a bioluminescent reporter (cFLuc-DEVD) in which luciferase activity is dependent on caspase-3 cleavage[21,22]. To test this construct, we treated HeLa cells stably modified with the cFLuc-DEVD construct, with a potent apoptosis inducer and measured luciferase activity in cell lysates. As anticipated, staurosporin treatment resulted in a strong increase in light emission reflecting the increase caspase 3 activity (Fig. 1A). To determine whether this reporter could be used to monitor T cell-induced cell death, we generated surrogate beta-cells by genetically modifying HEK 293T cFLuc-DEVD cells with a lentiviral vector containing the full-length PPI cDNA driven by a CMV early enhancer and promoter. Transduction of HEK 293T cells resulted in expression of the PPI expression cassette as was evident from qPCR analysis (Fig. 1B). Proinsulin synthesis was verified by western blot analysis (Fig. 1C). Overnight incubation of these surrogate beta cells, with increasing amount of $PPI_{(SP15\text{-}24)}$-specific CTL led to increased light emission reflecting the CTL-induced apoptosis of the target cells. In contrast, luciferase activity was unaffected when unmodified HEK 293T cFLuc-DEVD cells were used as target (Fig. 1D). These data show that ectopic expression of PPI sensitizes HEK 239T cells to $PPI_{(SP15\text{-}24)}$-specific CTL cytolysis and validate the specificity of our assay.

The localization of PPI signal peptide within the ER and the TAP independent routing of the $PPI_{(SP15\text{-}24)}$ point to alternative degradation mechanisms [16]. The presence of three alanine residues at the C-terminal part of the PPI signal peptide represents a high-affinity binding motif for the ERAP1 hydrophobic pocket and the two leucine residues in position 13-14 makes this region a suitable substrate for ERAP1 trimming[19]. To test whether ERAP1 plays a role in the generation of the $PPI_{(SP15\text{-}24)}$ peptide, we transduced surrogate beta reporter

cells with ERAP1-specific shRNA containing lentivirus (MOI=1). Expression of shERAP1 led to 90% reduction of ERAP1 mRNA compared to a non-specific short hairpin (Fig. 2A) without affecting ERAP2 gene expression (data not shown). Of note, the reduced ERAP1 expression in our assay had no impact on insulin gene expression or HLA-ABC surface expression (Fig. 2B, C). Downregulation of ERAP1 reduced $PPI_{(SP15-24)}$-specific CTL-mediated target cell destruction, as a 60% reduction of caspase-3 induced luciferase activity was observed at a 1:10 Target:Effector ratio (Fig. 2D). Taken together, our data confirm the implication of ERAP1 in the maturation of the non-conventional $PPI_{(SP15-24)}$ epitope from human PPI[18].

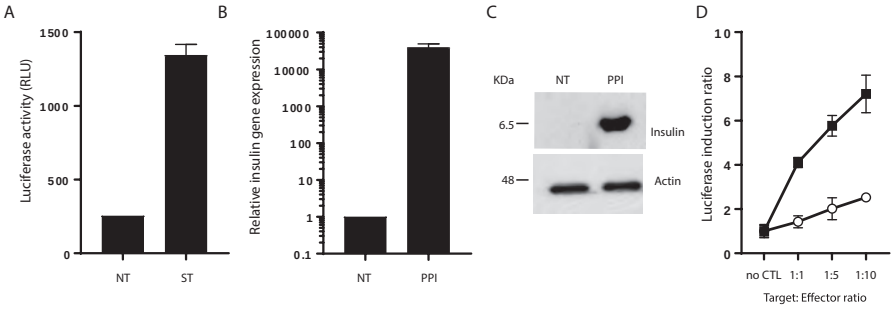

Figure 1: Characterization of caspase 3-inducible reporter and surrogate beta cells. (A) Transduced HeLa cells with cFLuc-DEVD reporter were exposed to 100nM staurosporine (ST) for 1 hr compared to non-treated controls (NT). Results are shown in relative light units (RLU). The results depict the average of 3 experiments. (B) Quantitation of PPI expression in HEK 293T/GFP cells and HEK 293T cells transduced with LV-CMV-PPI-bc-GFP by qPCR (MOI=1). Quantitative PCR analysis has been performed in triplicate and GAPDH corrected. (C) Western blot analysis of PPI expressing cells using an insulin-specific antibody and as control a human actin-specific antibody. (D) HEK293T/GFP cells expressing cFlucDEVD (open symbols) and HEK 293T cells expressing PPI and cFlucDEVD 293T cells (black symbols) were cocultured 16h with increasing numbers of PPI(SP15-24)-directed CTL.

Proinflammatory cytokines play a central role in triggering autoimmunity by inducing ER stress in beta cells[7,23-25]. To evaluate the importance of inflammation-induced ER stress on ERAP1 expression in human beta cells, we treated EndoC-βH1 cells with a mixture of the proinflammatory cytokines IFNγ and Il-1β. ERAP1 was upregulated in the presence of cytokines and this was associated by the upregulation of XBP1s, an important transcription factor induced upon ER stress (Fig. 3A). Similar results were obtained in primary human pancreatic islets (Fig. 3B). Increased ERAP1 gene expression was mirrored by ERAP1 protein levels (Fig. 3C). Activation of the endoribonuclease

IRE1α upon ER stress resulted in the processing of the XBP1 mRNA by and the removal of 26bp to generate an active XBP1s transcription factor. Inhibition of IRE1α by MKC3946, led to reduced XBP1 splicing [26]. Accordingly, co-treatment with MKC3946 dampened cytokine-induced ERAP1 gene expression in EndoC-βH1 cells (Fig. 3D)

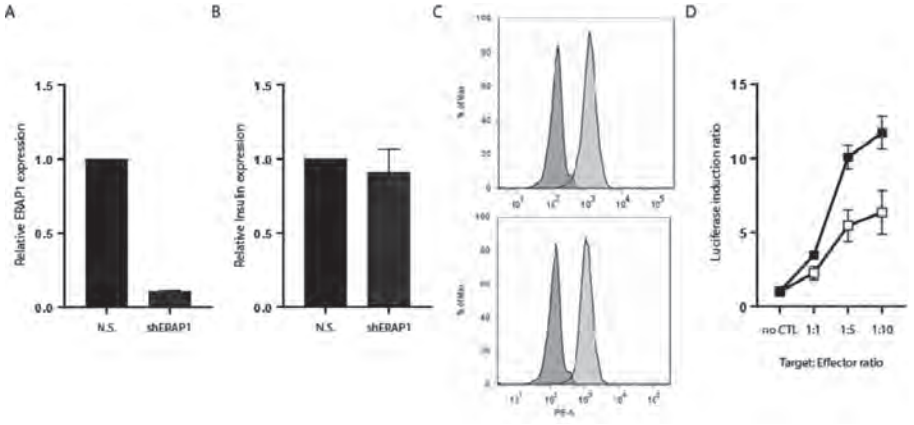

Figure 2: ERAP1 down-regulation by shRNA lentiviruses reduce CTL-mediated killing. A) PPI cFLuc-DEVD HEK 293T cells transduced with lentiviruses containing a non-specific shRNA cassette (N.S.), or a shRNA cassette directed against ERAP1 (shERAP1). Four days post-transduction, cells were analyzed for ERAP1 expression by qPCR. (B) Expression of insulin gene in shERAP1 and non-specific shRNA (N.S.) cells assessed by qPCR. Quantitative PCR analysis has been performed in triplicate and GAPDH corrected. (C) HLA class I surface expression of non-specific shRNA (upper panel) and shERAP1 modified PPI cFLUC-DEVD HEK 293T cells (lower panel). Non stained cells are used as control (dark grey). (D) Killing assay performed on non-specific shRNA (solid squares) and shERAP1 (open squares) expressing PPI cFLUC-DEVD HEK 293T cells after 16 hr co-culture with increasing amounts of PPI(SP15-24) directed CTL. Luciferase activity in absence of CTLs was arbitrary set to 1. The results are shown as fold induction.

IRE1α has been shown to play a critical role in the control of beta cell dysfunction and death via degradation of miR-17-5p and the consequent activation of thioredoxin-interacting protein (TXNIP) [27]. Target sequence analysis of miRNAs expressed in beta cells, revealed 6 potential miRNAs that regulate ERAP1 gene expression[28] (Fig. 4A). All these miRNAs belonged to the miR-17 miRNA family, indicating a conserved miRNA regulatory mechanism (Table 1). In line with these predictions, stimulation of human beta cells with proinflammatory cytokines showed a correlation between increased ERAP1 and XBP1s expression and decreased miR-17 expression as assessed by qPCR (Fig. 4B). Of note, endogenous insulin expression remained unchanged upon cytokine stimulation. To test whether miR-17 has a direct effect on ERAP1, we generated luciferase reporter constructs in which a fragment of the ERAP1

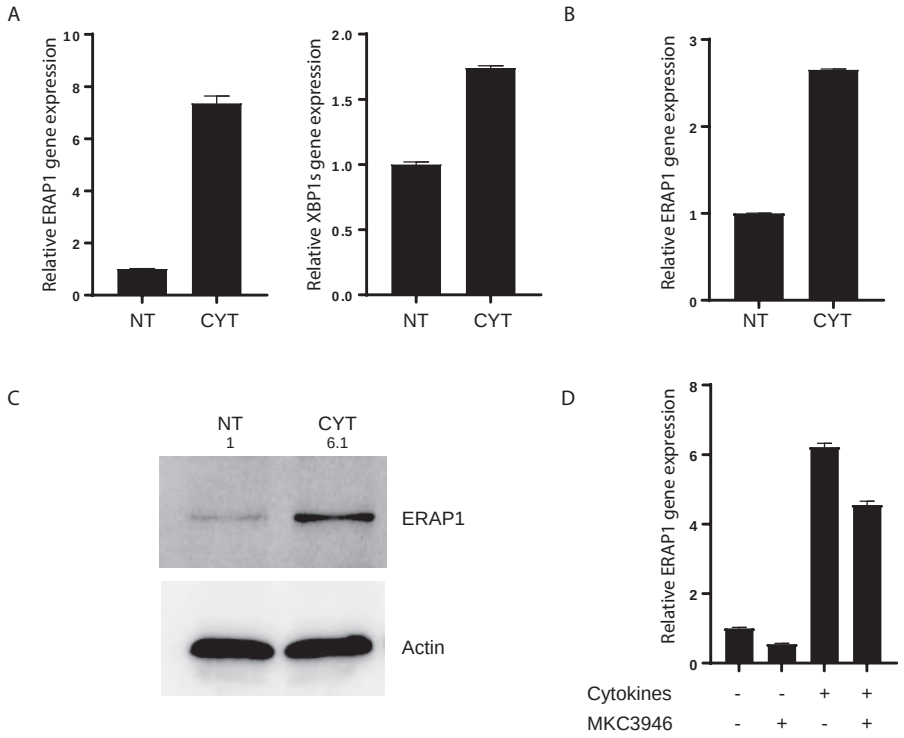

Figure 3: Effect of ER stress induction on ERAP1 expression. A) Relative ERAP1 (left panel) and XBP1s (right panel) expression in EndoC-βH1 cells after 24h treatment with IFNγ and IL-1β (CYT) compared to non-treated cells (NT) analyzed by qPCR. B) Relative ERAP1 expression in primary human pancreatic islets after exposure to proinflammatory cytokines for 24h (CYT) compared to non-treated islets (NT). C) Western blot analysis of EndoC-βH1 lysates for ERAP1 protein expression (upper panel). Actin was used as loading control (lower panel). Quantification has been performed by ImageJ and corrected for actin. D) Relative ERAP1 gene expression in EndoC-βH1 cells after cytokines stimulation in presence or absence of MKC3946 (10uM). Quantitative PCR analysis has been performed in triplicate and GAPDH corrected.

3'UTR containing the potential miR-17 binding site was cloned between the stop codon and the poly-A of the luciferase coding sequence. A UTR mutant in which two mutations were introduced to prevent miR-17 binding was generated and used as control in our assay. Transient expression of miR-17 in HEK 293T luciferase reporter reduced luciferase activity in presence of native UTR sequence, while luciferase activity cells transfected with a mutated reporter UTR sequence remained unchanged compared to control miR-1 (Fig. 5A). These results confirm that miR-17 can directly regulate ERAP1 expression. Supporting these findings, the forced expression of miR17 in human beta cell blunted proinflammatory cytokine-induced upregulation of ERAP1 (Fig. 5B).

Altogether, our data demonstrate a link between proinflammatory cytokine-induced ER stress and ERAP1 via IRE1α-mediated miR-17 suppression in the control of preproinsulin signal peptide trimming, autoantigenic epitope generation, and CTL-mediated target cell destruction.

Table 1: Prediction of ERAP1 targeting miRNAs expressed in human pancreatic beta cells.

miRNA	ERAP1-target motif	Folding energy (Kcal/mol)
Hsa-miR-20b-5p	CAAAGUGCUCAUAGUGCAGGUAG	-16.20
Hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	-16.20
Hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	-16.00
Hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	-16.00
Hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	-14.30
Hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU	-15.50

ERAP1-miRNA motif and the energy folding characteristic of the miR-17 family on the ERAP1 UTR region. Data presented are according to in silico predictions performed on https://cm.jefferson.edu/rna22/Interactive/RNA22Controller.

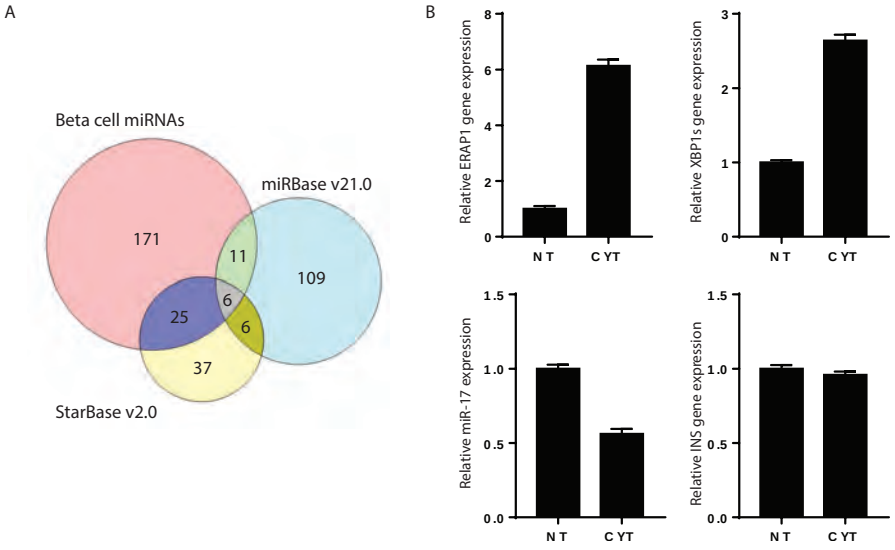

Figure 4: Correlation between ER stress-induced miR-17 and ERAP1 gene expression. A) Venn diagram showing the overlap between miRNAs found in beta cells and predicted ERAP1 targeting miRNAs according to miRbase V21.0 and StarBase V2. B) Relative expression of ERAP1 (top left) XBP-1s (top right), MiR-17 (bottom left) and INS (bottom right) in EndoC-βH1 cells after cytokine stimulation (CYT) compared to non-treated cells (NT). Quantitative PCR analysis has been performed in triplicate. ERAP1, XBP1s, and insulin are GAPDH corrected. MiR-17 expression has been corrected for RNU6.

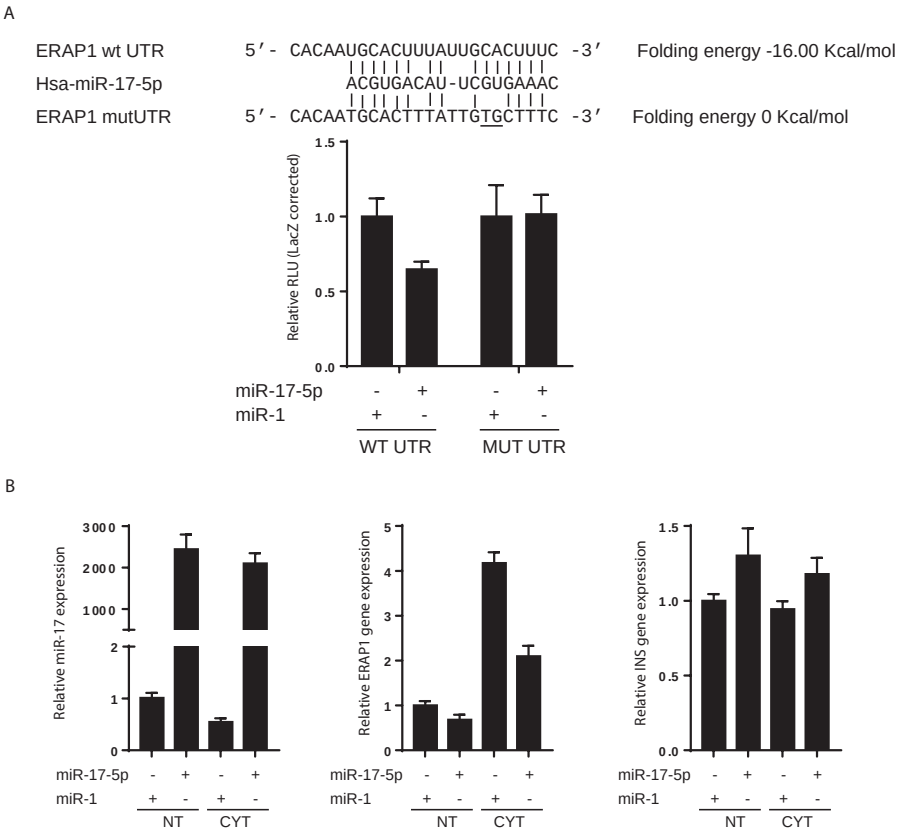

Figure 5: MiR-17 regulates ERAP1 gene expression. A) Schematic representation of the miR-17 targeting sequence in the 3-UTR of ERAP mRNA (wt UTR) and the mutated ERAP1 UTR (mut UTR) in the luciferase reporter constructs with the predicted folding energy (top). Relative luciferase activity after co-transfection of miR-1 (negative control) or miR-17 with ERAP1 wt UTR luciferase construct or ERAP1 mut UTR in 293T cells (bottom). Data are corrected for LacZ activity. B) Relative expression of miR-17 (left panel), ERAP1 (middle panel) and INS (right panel) in EndoC-βH1 following miR-17 or control miR-1 transfection in the presence or absence of cytokine stimulation. Quantitative PCR analysis has been performed in triplicate. ERAP1 and insulin are GAPDH corrected, miR-17 expression has been corrected for RNU6.

DISCUSSION

Here, we demonstrate a new regulatory mechanism connecting ER stress induction and increased beta cell exposure to the immune system.The signal peptide of PPI represents a major source of antigenic peptides derived from insulin gene products [15,16]. Increased ERAP1 expression in isolated human islets and human beta cells upon IFNγ/IL1β stimulation would lead to an increased signal peptide processing and beta cell destruction during inflammation in

T1D patients. While *in vitro* trimming assays have pointed out a peptide length limitation for ERAP1 cleavage (the upper length limit was defined at 16 residues[19]), our data corroborate previous results on the implication of ERAP1 in $PPI_{(SP15-24)}$ processing and presentation [18]. The reduced apoptosis upon ERAP1 knockdown in surrogate beta cells after coculture with $PPI_{(SP15-24)}$-specific CTLs is in line with the reduced expression of T cell activation marker (MIP-1β) observed by Kronenberg-Versteeg *et al*. after targeting ERAP1 by siRNA in their assay [18]. Intriguingly, based on the essential role of peptide trimming for proper presentation of many epitopes, a decrease expression of HLA class I would seem conceivable upon down-regulation of ERAP1. However, transduction of shRNA directed against ERAP1 had no effect on surface expression of HLA. The exact function of ERAP1 in controlling MHC class I expression remains unclear. While ERAP1 knockdown by siRNA in HeLa cells was shown to slightly reduced HLA class I expression, this has not been observed for $H\text{-}2K^b$ surface expression in mice [29,30].

ERAP1 was initially described as IFNγ responsive gene[31]. Yet, cotreatment of proinflammatory cytokines with MKC3946 demonstrates that the IRE1α pathway participates to the regulation of ERAP1 gene expression and reveals a direct link between ER stress and ERAP1 gene expression. Several miRNAs have been implicated in the response to stress [32], among these miR-17 has been shown to be a master regulator of beta cell apoptosis by controlling the thioredoxin-interacting protein (TXNIP)[27,33]. Inhibition of TXNIP prevents diabetes development in mouse models fort T1D and T2D, however the role of TXNIP in beta cells is complex. TXNIP modulates the cellular redox state and participates to the induction of oxidative stress and consequently the induction of apoptosis by activation of caspase 2 and release of cytochrome C, as well as the amplification of insulitis by activating the NLRP3 inflammasome, activation of caspase 1, and maturation of Il1β [34-36]. While TXNIP regulation was demonstrated to be independent of ATF6, PERK and IRE1α were shown to participate to TXNIP transcriptional and post-transcriptional control, respectively [37]. Whether the other arms of the UPR are involved in ERAP1 regulation and expression, as for TXNIP, needs to be determined.

In this study, we confirmed the complexity of antigenic peptide generation originating from signal peptide domain of the human preproinsulin. The implication of the IRE1α/miR-17 pathway in regulating the resident ER protein trimming underscores the key role played by ER stress in the development of autoimmunity. The central role for IRE1α/miR-17 in beta cell dysfunction

and apoptosis via upregulation of TXNIP and autoantigen processing via upregulation of ERAP1 designate IRE1α as an interesting therapeutic target in diabetes. This intervention opportunity is supported by a recent finding of reversed autoimmune diabetes in NOD mice after IRE1α inhibition[38].

MATERIALS AND METHODS

DNA Constructs

pLV-CMV-cFLuc-DEVD was generated by insertion of a PmeI/PmeI fragment from the pcDNA3.1-cFLuc-DEVD[21] into the backbone of pLV-CMV[39] restricted with EcoRV. Preproinsulin cDNA was obtained from reverse transcriptase reaction of total human islets RNA extraction using the following primers: *PPI-Full(Fw)*: ATG GCC CTG TGG ATG CGC CTC CTG CCC; *PPI-Full(Rv)*: GTT GCA GTA GTT CTC CAG CTG GTA GAG GGA GCA. LV-CMV-PPI was generated by insertion of the coding region of the preproinsulin cDNA into pLV-CMV-bcGFP. For miRNA reporter construct the following primers were annealed and cloned into pMIR-REPORT (Thermofisher Scientific) open with PmeI. ERAP UTR Fw: 5'-gta att tga ata tag aca caa tgc act tta ttg cac ttt caa ttc tta taa agc; ERAP UTR Rv 5'-GCT TTA TAA GAA TTG AAA GTG CAA TAA AGT GCA TTG TGT CTA TAT TCA AAT TAC; ERAP UTR mut Fw 5'-gta att tga ata tag aca caa tgc act tta ttg tgc ttt caa ttc tta taa agc; ERAP UTR mut Rv 5'-GCT TTA TAA GAA TTG AAA GCA CAA TAA AGT GCA TTG TGT CTA TAT TCA AAT TAC. The constructs were verified by sequencing.

Cells and reagents

293T (HLA-A2 positive) were grown in high glucose DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (Gibco BRL) and penicillin/streptomycin at 37C, 5%CO2. $PPI_{(SP15-24)}$ directed CTLs were maintained in IMDM supplemented with 10% human serum, IL-2 and IL-15 and restimulated every 14 days with JY cells (pulsed with 10mg/ml PPI15-24 peptide) at 1:5 ratio in presence of IL-2 and IL-15. PPI15-24 was synthesized using solid-phase Fmoc chemistry, analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry for purity and identity. EndoC-βH1 cells, kindly provided by Dr. Raphael Scharfmann (Paris Descartes University, France), were maintained in low glucose DMEM supplemented with 5.5 µg/ml human transferrin, 10 mM nicotinamide, 6.7 ng/ml sodium selenite,

50 μM β-mercaptoethanol, 2% bovine serum albumin fraction V, 100 units/ml Penicillin and 100 μg/ml Streptomycin. Cells were seeded in ECM, fibronectin pre-coated culture plates.

Inflammatory stress was induced by a mixture of 1000 U/ml IFNγ and 2 ng/ml IL1β for 24 h. Staurosporine was used at 100nM for 1h and Thapsigargin was used at 100nM for 24h. MKC3946 inhibitor was used at 10uM for 24h in our assay.

MiRNA transfection

Transient transfection of miRNA mimics were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. EndoC-βH Cells were transfected in a 24-well plate, using a final concentration of 50 nm miRNA precursor, Hsa-miR-17-5p precursor (PM12412, Ambion) or premiR Hsa-miR-1 (Ambion), was used. Experiments were continued 24 h post transfection. For the validation of miRNA targeting sites, 125 ng pMiR-luc-ERAP1-wtUTR or pMIR-luc-ERAP1-mutUTR, 5 ng pLacZ and 50 nM miRNA precursor Hsa-miR-17-5p precursor or premiR Hsa-miR-1 were used per well (96-well plate) to modify 293T cells. Transfections were performed in triplicate and cells were analysed 24 hours post transfection.

Lentiviruses production and transduction

The vectors were produced as described previously[39]. Briefly, the lentiviral backbone containing the gene of interest and the three helper plasmids (encoding HIV-1 gag–pol, HIV-1 rev, and the VSV-G envelope protein) were co-transfected overnight using the calcium phosphate method into 293T cells. The medium was refreshed and viruses were harvested after 48 and 72 h, passed through 0.45-um filters, and stored at -80°C. Virus was quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corp., New York, NY, USA) as described[40]. Then, viral supernatants were added to fresh medium supplemented with 8 μg/ml Polybrene (Sigma), and the cells were incubated overnight. The next day, the medium was replaced with fresh medium. Transduction efficiency was analyzed 3 to 6 days post transduction.

ERAP1 downregulation

shRNA lentiviral constructs for ERAP1 knockdown were obtained from the Mission shRNA library (Sigma-Aldrich clones TRCN060539; TRCN060540; TRCN060541; TRCN060542). Based on preliminary assays to assess knock-down efficiency (data not shown), we selected the TRCN060542 clone for further use.

The shERAP1 encoding lentivirus was produced as described above.

FACS analysis

Cells were washed in PBS containing 0.1% BSA and subsequently incubated in 100 µl of PBS/0.1% BSA with the appropriate amount of antibody for 60 min at 4°C in the dark. Cells stained with the proper isotype were used as negative control. HLA-A2 and HLA-ABC-FITC were purchased at BD Pharmingen Inc. (San Diego, CA, USA). For flowcytometry, 10,000 events were counted on a FACS LSRII (BD Pharmingen Inc., San Diego, CA). Data analyses were performed using FlowJo software.

RT-PCR / qPCR

Total RNA was extracted from cultured cells using Trizol reagent following manufacturer's instructions. Isolated RNA was quantified using a Nanodrop 1000 spectrophotometer. Approximately 500 ng RNA was reverse transcribed using Superscript RT II kit (Invitrogen, Karlsruhe, Germany). Expression of the genes interest was detected using the following primers: Insulin Fw GCA GCC TTT GTG AAC CAA CA, Insulin Rv CGG GTC TTG GGT GTG TAG AAG; ERAP1 Fw GAA AAC CAT GAT GAA CAC TTG G, ERAP1 Rv CCA CCT CTT CTG GGA GGA TGA G; GAPDH Fw ACA GTC AGC CGC ATC TTC TT, GAPDH Rv AAT GAA GGG GTC ATT GAT GG, XBP1s Fw 5'-CTGAGTCCGCAGCAGGTG-3', XBP1s Rv 5'-GAGATGTTCTGGAGGGGTGA-3'; ATF3 Fw 5'-GTGCCGAAACAAGAAGAAGG-3', ATF3 Rv 5'-TCTGAGCCTTCAGTTCAGCA-3'; CHOP Fw 5'-GACCTGCAAGAGGTCCTGTC-3', CHOP Rv 5'- CTCCTCCTCAGTCAGCCAAG-3'; Polymerase chain reactions were performed on a PTC-200 (Biozym, Landgraaf, The Netherlands) using the following conditions: 94°C for 5 min; 35 cycles of 30" at 94°C, 30" at 60°C, and 1.5 min at 72°C; 10 min at 72°C. Real-time PCR were performed in triplicate using the SybrGreen master mix kit (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands) and an Applied Biosystems Step One Plus. Comparative ΔΔct values were performed using GAPDH gene as reference. Values are represented as mean ± standard error.

For microRNA quantification, total RNA was reverse transcribed using Taqman microRNA Reverse transcription kit (Applied Biosystems) and detected using Hsa-miR17 Taqman microRNA assays (PN4427975, Applied Biosystems) and TaqMan 2x Universal PCR Master Mix, no UNG (applied Biosystems) according to manufacturer's instructions. MicroRNA expression was normalized

to RNU6 using the following primers: RNU6 Fw 5'-CTCGCTTCGGCAGCACA-3'; RNU6 Rv 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot analyses

Cells were lysed in RIPA lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, SDS 0.1%, 0.5% DOC, 1% NP40, with proteases inhibitors). For insulin staining, proteins extracts were loaded on 17% acrylamide/bis acrylamide SDS page gel, and after electrophoresis, transferred to Immobilon-P transfer membrane (PVDF); Millipore, Etten-Leur, The Netherlands and treated with anti-insulin (1:1,000; Santa Cruz SC-9168), and anti-actin (1:5,000, clone C4; ICN Biomedicals, Inc., Zoetermeer, The Netherlands) sera. For ERAP1 analyses, proteins extracts were loaded on 10% acrylamide/bis acrylamide SDS gel. After blotting, the membranes were stained with anti-ERAP1 (Santa Cruz B10; Sc-271823).

CTL killing assay & luciferase assay

2.10^4 to 5.10^4 modified HEK 293T cells were plated in 96 well plate and exposed for 16h to increasing amounts of PPI directed CTLs. After overnight coculture, media was removed and cells were lysed in luciferase lysis buffer [125 mM Tris/HCl, pH 7.8, 10 mM CDTA, 10 mM DTT, 50% (v/v) glycerol, 5% (v/v) Triton X-100]. Light emission was determined using Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity from cells in absence of CTL was used as reference and arbitrary set to 1.

In silico analyses and statistical analysis

Data are presented as mean ± SEM. Calculations were performed using GraphPad Prism 7.

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CHAPTER 3

Neoantigens in type 1 diabetes

3.1 Neoantigens and microenvironment in Type 1 diabetes: lessons from antitumor immunity

3.2 A roadmap of the generation of neoantigens as targets of the immune system in type 1 diabetes

CHAPTER 3.1

Neoantigens and microenvironment in Type 1 diabetes: lessons from antitumor immunity

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ABSTRACT

Type 1 diabetes (T1D) is characterized by the selective and progressive destruction of insulin-producing beta cells by the immune system. An incomplete thymic selection against self-reactive islet antigens partly explains how these T cells reach the periphery and become diabetogenic. Increasing evidence suggest that beta cells themselves also participate to their own demise by generating neoepitopes that could be recognized by the immune surveillance machinery. In this regard, these T cells eradicate self-tissue by mechanisms analogous to a classical antitumor response. Cancer immunotherapy has exploited mutations and transcriptional and translational errors to trigger a specific antitumor response. In this opinion article, we aim at merging insight in antitumor immunology and autoimmunity to reveal processes that had previously been ignored to create beta cell-specific neoantigens.

Trend box

- The microenvironment during beta cell destruction in T1D resembles a prototype effective antitumor response, that is, clearance of abnormal, dysfunctional or potentially harmful cells.
- Modified proteins that escape thymic education and peripheral tolerance provide a new range of candidate neoantigens as targets in the pathogenesis of T1D, similar to mutated and tumor specific proteins in antitumor immunity.
- Identification of modified epitope-specific T cells may serve as biomarkers in the early detection of dysfunctional beta cells, thereby guiding early immune intervention to avoid progressive beta cell immunogenicity and destruction.

SIMILARITIES BETWEEN T1D AUTOIMMUNITY AND ANTITUMOR IMMUNITY

During thymic education, the majority of autoreactive T cells are eliminated by apoptosis while lower avidity T cells may escape negative selection in the thymus and reach the periphery [1]. Naïve islet autoreactive T cells are detectable in both T1D patients and non-diabetic subjects [2]. Activation of these T cells will require priming by the target tissue in dialogue with the immune system. Even after activation, peripheral islet autoreactive effector T cells will usually remain under control by various immune regulatory mechanisms including regulatory T cells (Tregs) [3,4]. There is increasing evidence that local inflammation combined with a genetic predisposition contributes to the break in immune tolerance, leading to insulitis and destruction of beta cells [5,6]. Yet, the circumstances under which these islet-autoreactive T cells become activated and specifically target beta cells in T1D patients still largely remains to be clarified.

Beyond imperfect thymic education of immune reactivity against native self-proteins, it is conceivable that dysfunctional beta cells either as cause or consequence of T1D etiology generate and accumulate aberrant, modified neo-autoantigens, which lack both central and peripheral immune tolerance. These neoantigens may prime, activate, maintain, exacerbate, and accelerate T1D, either directly by affecting target cell viability and integrity, or indirectly by the generation of neoepitopes priming diabetogenic T cells.

Despite many differences, including time and magnitude of the immune response between anti-tumor immunity and beta cell autoimmunity, we contend that the inflammatory signals surrounding the islets of Langerhans during insulitis (Box 1) resemble the signals identified at the vicinity of tumor cells during successful anti-tumor immune surveillance. In T1D, environmental stress and other perturbations in and around pancreatic beta cells may also disturb the cellular equilibrium and affect high fidelity transcriptional, translational and post-translational processes generating neoantigens to which the immune system is trained to act upon[6]. Cancer immunity and immunotherapy have exploited these errors and modifications to trigger specific immune responses against tumor-associated self-antigens[7]. The participation of similar post-transcriptional and (post-) translational errors in the process of beta cell destruction still deserves investigation but point to a striking similarity in the immunopathogenesis of T1D and effective anti-tumor immunity (Figure 1).

Here, we provide a comprehensive overview of post-transcriptional and (post-) translational processes currently known to occur in autoimmune diseases and in tumor immunology. We describe the different pathways affected by an inflammatory environment leading to generation of tumor-specific neoantigens to guide discovery of new, relevant islet epitopes that act as surrogates of diabetogenesis and insulitis. This may improve immune monitoring of disease progression and therapeutic intervention.

Box 1. Why is beta cell destruction selective? Influence of islets microenvironment

Islets of Langerhans are embedded in exocrine pancreatic tissue. In addition to their role in secreting digestive enzymes, autopsy studies suggest that the exocrine pancreas is involved in the development of autoimmunity by supporting trafficking of immune cells to the islet [89], even though the rate of exocrine inflammation was much less in pancreatic biopsy studies in living newly diagnosed T1D patients [90].

Strikingly, beta cells remain the primary target of destruction by the immune system. Several studies have shown that endocrine cells differ in their resilience to stress condition. Under pathophysiological mimicking T1D, beta cells were shown to be more sensitive than alpha cells to proinflammatory cytokines stimulation [91], and similar results were obtained after metabolic stress [92]. Endocrine cells seem differentially equipped to stand an environmental insult. The intrinsic properties of beta cells being insulin factories might explain this sensitivity to environmental changes [93]. The high oxidative stress with which beta cells have to deal with, combined with HLA class I hyperexpression observed in T1D that may even precede insulitis, may lead to T cell infiltration and beta-cell specific destruction by autoreactive T-cells [16]. Yet, other endocrine cells within the islet may participate in the dialogue with the immune system without apparently being targeted by the immune system. The role of alpha cells, for example, has been largely underestimated. The precise architecture of human islets interconnecting alpha and beta cells fosters a direct crosstalk. Pioneer studies from Unger and Orci in the 1970s demonstrated the importance of glucagon in T1D, suggesting T1D to be a 'bihormonal' disease characterized by reduced insulin and high glucagon levels [94]. Alpha cells could be a missing link in the communication chain between immune system and beta cells. The recent demonstration that alpha cells represent an intra-islet source of glucagon-like peptide-1 (GLP-1), known for preventing beta cell apoptosis and promoting beta cell function [95], combined with the recent demonstration that IL-6 enhances GLP-1 release by alpha cells, strengthens the concept of a direct and

intimate relation between the immune compartment, alpha cells and beta cells that may even be invigorated during inflammation [96].

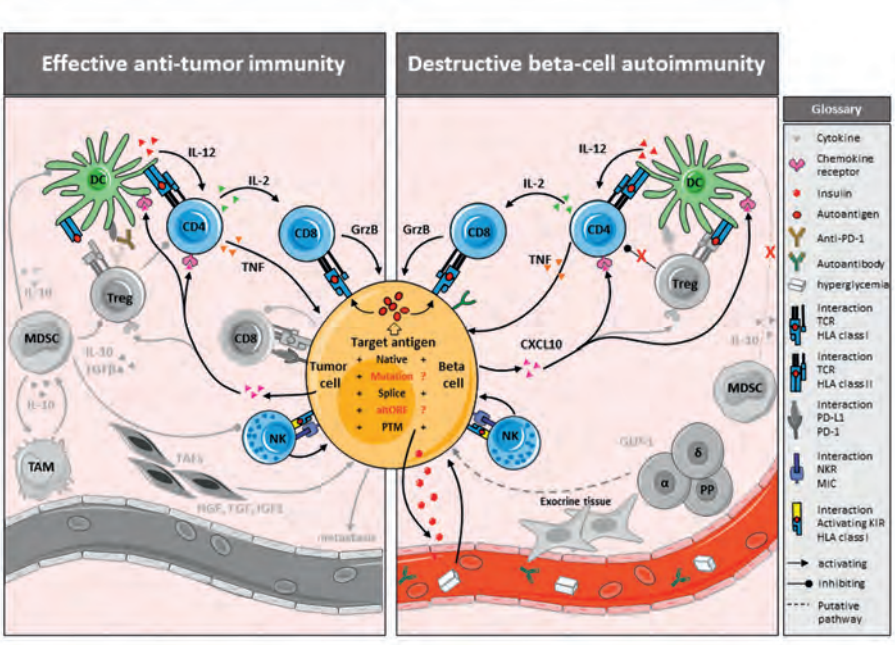

FIGURE 1: Similarities in inflammatory microenvironment between immune-mediated destruction of tumor and beta cells. The proinflammatory milieu in effective antitumor immunity highly resembles pathogenic immune responses in type 1 diabetes (T1D). Colored components are effectively engaged in target cell destruction. The tumor microenvironment otherwise suppressing antitumor immunity by downregulation of human leukocyte antigen (HLA) class I on tumor surface and recruitment of myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and tumor-associated fibroblasts (TAFs) sustaining tumor survival, and promoting tumor cell growth and metastasis (left panel, grey tones) is outweighed by a proinflammatory immune infiltrate and adaptive immunity against the target cancer cells (colored). Destruction of pancreatic beta cells in T1D follows similar proinflammatory processes (right panel, colored tones). We propose that lessons from a deleterious tumor microenvironment may guide studies investigating the roles of MDSCs, regulatory T cells (Tregs), and contribution of the beta cell microenvironment (other endocrine islet cells [alpha, delta, PP] and exocrine tissue) that may control pathogenic beta cell destruction (broken arrows). At the heart of target cell destruction, the adaptive immunity involves recognition of target cell antigens. Recent insight points to a role of modified tissue antigens, in addition to native autoantigens in both the pathogenesis of type 1 diabetes and effective antitumor immunity or immunotherapy. Abbreviations: DC, dendritic cell; KIR, killer cell immunoglobulin-like receptor; NK, natural killer cell; TNF, tumor necrosis factor.

BETA CELL MICROENVIRONMENT VERSUS TUMOR MICROENVIRONMENT

Environmental factors are implicated in both T1D etiology and effective anti-tumor immunity. The resulting inflammation initiates progressive destruction of the target cells (beta cells and tumor respectively) by promoting the generation of immune responses against self-proteins via infiltrating dendritic cells.

In response to inflammatory environmental signals, both beta cells and tumor cells are in dialogue with the immune system by local release of proinflammatory cytokines (IL-1β)[8,9], chemokines (e.g., CCL2, CXCL10)[10-12] and by the generation of neoantigens recognized by tumor specific or autoreactive T cells [13,14] (Figure 1). Increased Human Leukocyte Antigen (HLA) expression is one of the first features of islet distress, apparently preceding insulitis in case of T1D [15]. While tumors may develop ways to evade immune detection (e.g. down regulation of HLA class I, sialic acid expression, and direct inhibition of lymphocytes [16,17]), in beta cells the HLA class I hyper-expression in combination with production of chemo-attractants amplifies the visibility for immune surveillance and magnitude of the immune response [10,11]. This, together with signals generated by invading immune cells (e.g. CD8 and CD4 T cells), may contribute to the formation of a microenvironment comparable to an effective anti-tumor response.

In case of progressive tumors, the microenvironment contains other cells that contribute to tumor growth, such as tumor-associated fibroblasts (TAFs) secreting TGFβ, FGF, IGF1 or other pro-angiogenic factors (VEGF, PDGF) [18,19], myeloid-derived suppressor cells (MDSCs) that prevent cytotoxic T cell activity, and recruitment of tumor-associated macrophages (TAMs) involved in escaping anti-tumor mechanisms by secreting immunosuppressive cytokines (e.g. IL10) [20,21]. Macrophages can be found in insulitic lesions [22] but while TAMs migrate to the inflammatory site, phenotype characterization of pancreatic macrophages in mouse suggest that M1 islet resident macrophages can be involved in beta cell destruction [23]. MDSCs have recently been reported to be increased in peripheral blood of T1D patients, whereas their frequency was decreased in the islet microenvironment in diabetic NOD mice, implying an underlying defect in immune suppression [24]. Natural thymus derived regulatory T cells (nTregs) ($CD4^{+}CD25^{high}$ with demethylated FoxP3) impair

effective anti-tumor responses[25]. Immunotherapy targeting CD25 releases curative anti-tumor T cell immunity[26]. Studies in NOD mice suggested that progression to autoimmune diabetes was associated with a reduction of nTregs[27]. nTregs have not been detected in human insulitis yet, in marked contrast to mouse models this lack of 'natural' immune regulation would certainly aid to the immune imbalance favouring autoimmunity and T1D. In addition, while the frequency of circulating nTregs is similar in healthy and T1D subjects, their suppressive quality is reduced in T1D, whereas effector T cells seem resistant to suppression by nTregs from T1D donors [3,28,29]. Tissue specific Tregs reactive with islet autoantigens have also been reported to be less frequent in T1D than healthy subjects [3]. These islet specific Tregs may exert their suppressive activity in healthy and slow-progressive T1D subjects by unorthodox interaction of their T cell Receptor (TCR) with the islet peptide/HLA class I molecule [30].

SOMATIC MUTATIONS IN DISEASE PROGRESSION

The vast majority of all cancers are linked to somatic mutations in association with environmental factors. Bacterial infections are often associated with gastric, bladder or colon cancer, infections with hepatitis viruses have been associated with increased hepatocellular carcinoma, EBV infection with Hodgkin's lymphoma, while non-microbial environmental factors (environmental toxins, ultraviolet light or smoke) have also been shown to promote carcinogenesis (lung cancer, thyroid cancer, melanoma) [31,32]. These somatic mutations can accumulate during cell division but remain, usually without tumorigenic consequences. Yet, in rare cases, these mutations affect important cell regulator elements leading to anarchic cell division and apoptosis inhibition causing tumor development. Fortunately, the immune system is endorsed with highly efficient and sensitive immune surveillance specifically targeted to discover subtle differences in tissues distressed by infection or carcinogenesis. While some mutations contribute to tumorigenesis by activation of oncogenes[33], others play an important role in anti-tumor-immunity by generating specific tumor neoantigens that may trigger an immune response against cancer cells [34]. The low division rate of adult beta cells makes somatic mutations unlikely to happen during T1D development [35]. Similarly, somatic mutations

in TCR genes that could have led to a change in peptide affinity and specific recognition of beta cell self-peptide have not yet been reported.

ENVIRONMENT AND RNA EDITING

Beta cells in insulitis and tumor cells under immune attack are immersed in a similar environment, receiving similar inflammatory signals that lead to the development of autoimmunity or anti-tumor immunity (as detailed in figure 1). Next generation sequencing showed that most RNA, in healthy tissue, undergo alternative splicing by exon skipping or cryptic splicing sites leading to modification of the coding region [36]. Alternative splicing is an adaptive mechanism that participates to genome complexity. Proper introns removal from pre-mRNA is a complex mechanism involving the recognition of splicing sites by small nuclear ribonucleotide particles assembled as spliceosome complex. The still poorly understood regulation of these processes involves participation of cis-/trans-acting splicing proteins and is regulated by splicing enhancers/inhibitors located in upstream exons or introns [37]. During tumor development, an inflammatory environment can lead to perturbation of splicing regulatory factors generating cancer-specific transcripts different from the classical pool of mRNA expressed in healthy tissue [38]. Several studies conducted in ovarian, breast and colon cancer showed that over 50% of the transcripts were affected during tumor development [39]. Since each of these alternative transcripts can possibly generate a pool of neo-polypeptides (functional or not), the peptidome of the tumor cell can be both complex and tumor specific [40]. First evidence of these alternative splice variant epitopes was provided by transcriptome analysis of melanomas and the discovery of the tumor antigen MAGE-1, able to stimulate human T cells [41]. Initially identified in melanoma, MAGE-1 belongs to a gene family (MAGE-A; MAGE-B, MAGE-C) characterized by a large terminal exon that is silent in normal tissue but expressed in tumor cells [42]. This differential expression between healthy tissue and cancer made MAGE gene products a target of choice for antitumor immunotherapy.

In melanoma also incomplete splicing of the N-Acetyl glucosamyl transferase V with intron retention was observed in more than 50% of tumor cells whereas healthy tissue exclusively expressed fully spliced form. Again, this alternative splicing gave rise to the generation of unique epitopes encoded

by the intronic region recognized by CD8 T cells [43]. Although alternative transcripts are of interest as tumor progression biomarker, their relevance as target for immunotherapy remains questionable due to the heterogeneity of antigen expression in tumors; therefore tumor-specific vaccination might be inefficient with tumor cells lacking the antigen.

Recently, elevated alternative mRNA splicing events have been observed in inflamed human islet transcriptome studies providing evidence of beta cell neo-autoantigen generation during insulitis [44]. Although many splicing variants have been reported affecting cell function, the altered transcriptome in inflamed islets is a potential template for the generation of neoantigens and neoepitopes that subsequently can prime autoreactive T cells.[45].

Autoimmune disease reflects a combined loss of both central and peripheral tolerance leading to activation of autoreactive T cells. The differential protein expression between thymus and periphery of the proteolipid protein (PLP), is believed to contribute to multiple sclerosis (MS). While two transcript are expressed in the central nervous system, a full length PLP and a shorter variant lacking 35 amino acids encoded by a portion of exon 3 (DM20), only the short variant was detected in the thymus. Peripheral activation against sequence encoded by exon 3, leads to destruction of the myelin component in the central nervous system [46]. Similarly, differential expression of insulin, protein tyrosine phosphatase-like protein (IA-2) and Islet-specific glucose-6-P catalytic subunit-related protein (IGRP) in the thymus and the pancreas may also contribute to lack of central tolerance, creating a fertile environment for the development of T1D. In the thymus, IA-2 is produced as a protein lacking the transmembrane and the juxta-membrane domain encoded by exon 13. The identification of a T cell response against an epitope located in this domain demonstrates the lack of tolerance to IA-2 [47]. Also, while the full IGRP transcript is expressed in the pancreas, the thymus expresses forms lacking exon 2, 3 or 4. This differential expression participates to the incomplete tolerance towards IGRP in T1D and makes these regions, as well as the junction with other exons particularly immunoreactive [48,49]. Additionally, three extra splice variants, consequence of cryptic splicing sites in exon 5, have been identified specifically in the pancreas [48], leading to protein truncation, frameshifts and neoepitopes prone to immunogenicity. Strikingly, the relatively high frequency of cytotoxic CD8 T cells reactive with these neoantigens was similar in T1D and healthy controls, underscoring the lack of thymic education and central tolerance and

indicate that such T cells are part of the classical T cell repertoire [48]. Yet, their activity and rate of proliferation is increased in T1D subjects [50].

ENVIRONMENT AND TRANSLATION FIDELITY

Under stress, the cellular equilibrium may become disturbed, affecting high fidelity translational processes. So-called Defective Ribosomal Products (DRiPs) arising from translation of untranslated regions (UTR), ribosomal frame-shifting and alternative translation initiation, have been reported in tumor immunology to generate a unique class of tumor-associated antigens selectively expressed by malignant cells[51]. Interestingly, these translational products represent a pool of short lived polypeptides rapidly identified and degraded by the cellular quality control machinery that could represent a major source of HLA class I epitopes[51,52]. Among these, different reading frames of melanoma-overexpressed antigen (MELOE1), New York oesophageal squamous cell carcinoma 1 (NY-ESO-1), and L-antigen familiy 1 (LAGE-1) are common tumor-antigens, expressed in a variety of malignancies, and are highly immunogenic as tumor infiltrating lymphocytes (TILs) have been detected against multiple class I and class II epitopes in the circulation of cancer patients[53-55]. Currently, these tumor antigens serve as biomarkers and are explored as therapeutic targets in order to promote the natural immune response against these cells. Phase I/II trials with engineered human-derived T cells in patients with multiple myeloma demonstrated long-term engraftment, T cell infiltration in tumors, and retained T cell cytotoxicity [56].

Translation initiation and efficiency are highly sensitive to environmental conditions. Increased phosphorylation of eukaryotic initiation factor-2 (EIF2), due to upregulated kinase activity in response to stress, prevents the exchange of GDP for GTP, increasing the time required for scanning ribosomes to initiate translation [57]. In general, this leads to repressed protein synthesis, but for a subset of mRNAs involved in the regulation of the cellular stress response (ATF4, ATF5, GADD34 and CHOP), this enhances expression by increasing translation initiation at alternative AUGs. Proof for a role of endoplasmic reticulum (ER) stress in human insulitis was provided by immunostainings of pancreatic sections showing that islets from T1D donors display ER stress by increased levels of CHOP and BIP, compared to non-diabetic control tissues

[58]. Yet, little is known how ER stress affects the translation fidelity of beta cell specific proteins.

So far, the contribution of DRiPs as new source of neo-autoantigens has not been explored in T1D. In another autoimmune disease, a single report demonstrated the presence of autoreactive T cells directed against a frame-shift epitope from endogenous IL-10 in synovial fluid of a patient with Reiter's syndrome, where an autoimmune reaction triggered a response to a primary infection resulting in inflammatory arthritis [59].

ENVIRONMENT AND POST-TRANSLATIONAL MODIFICATIONS

Protein modification is tightly linked to extracellular events. For instance, tissue transglutaminase 2 (tTG2), whose activity is regulated by inflammatory signals, was shown to promote the epithelial-to-mesenchymal transition preceding tumor formation by switching E-to N-cadherin expression [60]. Also, tTG2 expression correlates with renal cell carcinoma progression and contributes to the development of chemoresistance in malignant melanoma [61].

Post Translational Modifications (PTM) may arise from enzymatic modifications (e.g. citrullination, glycosylation, deamidation) as well as non-enzymatic (spontaneous) modifications (e.g. methylation, carbamylation, oxidation, nitration) [62-65] and can result in a change in antigenic properties by single amino acid substitution, increasing HLA binding affinity thereby exposing cells to immune surveillance [65]. Deamidation of asparagine as the result of enzymatic cleavage of asparagine linked glycans by peptide-N-glycanase (PNGase) is a well-known source of tumor neoepitope formation in melanoma [66]. PTM of tyrosinase by deamidation presented the first proof of principle [67]. This modification did not alter binding to HLA but allowed for recognition by melanoma specific CD8 T cells. Formation of stable succinimide intermediates during deamidation, as well as non-enzymatic degradation of asparagine through UVA exposure, has also been shown to produce MHC class I binding neoepitopes [68,69].

Phosphorylation of cellular proteins is a key step in almost all intracellular signalling pathways. Nevertheless, dysregulation of phosphorylation, a hallmark of tumorigenesis, can create tumor specific phospho-peptides [70].

A recent study showed that over 60% of the identified phospho-peptides were tumor specific and can be presented on both HLA class I and class II molecules, creating opportunities for both CD4 and CD8 T cell involvement in cancer immunotherapy [71,72]. The amount of peptide potentially generated by tumors cells can also be amplified by post degradation processes (Box 2).

A variety of PTMs of islet autoantigens in T1D have also been reported in recent years and shown to be clinically relevant, as they were associated with T cell responses [65,73]. One of the first implications of PTMs in T1D was a disulphide-bond formed between two adjacent cysteine residues at position A6 and A7 of the insulin-A chain, which was recognized by autoreactive T cells isolated from T1D subjects [74]. Further research to PTMs of islet autoantigens revealed an increase tTG2 activity in isolated islets when maintained in pathophysiological conditions, while deamidation by tTG of potential islet epitopes increased the peptide binding affinity to high-risk HLA-DQ molecules [73,75]. $CD4^+$ T cells were found to a single and double deamidated form of the insulin B30-C13 epitope in new-onset T1D patients [73], which correlated with increased pro-inflammatory T cell responses. Beside modified insulin, multiple post-translationally modified GAD65-derived epitopes have been described [76]. A transglutamidated and a citrullinated GAD65 epitope were found and recognized by autoreactive $CD4^+$ T cells. In addition, these T cells were detected in significantly higher frequencies in peripheral blood of T1D patients than in healthy controls, while the quality of the immune response against this PTM was pro-inflammatory in T1D patients versus anti-inflammatory in non-diabetic subjects [76].

Box 2. Proteome diversity: The case of transpeptidation in T1D

Besides protein degradation, the proteasome is able to fuse peptides by a process called transpeptidation. Degradation of proteins by the proteasome results in the formation of an acyl-enzyme intermediate complex in which the C terminus remains attached to the catalytic core through an ester link. Upon hydrolysis, the hydroxyl group of the peptide is restored and the peptide released from the proteasome and loaded on MHC molecules without further modification [97]. However, depending on the stability of this intermediate, the fragment can undergo a nucleophilic attack by the N terminus of another newly cleaved peptide fragment, thereby joining two otherwise distant sections of a protein. In tumor immunology, CD8 T cells recognizing a peptide resulting from the junction of noncontiguous peptide fragments of melanocytic glycoprotein gp100 (PMEL17) were found infiltrating melanoma cells [97]. Similarly, hybrid peptides generated from reverse splicing [98], in which the

peptide fragments are fused in reverse order, and hybrid peptides generated from trans-splicing were identified, in which the origin of the joined splicing fragments are derived from different proteins [99]. The case of transpeptidation in T1D has recently been underpinned by the discovery of hybrid peptides of proinsulin fragments fused with chromogranin A or IAPP that became targets of islet infiltrating T cells in mice and men, reinforcing the potency of neoepitopes created by peptide fusion in the pathogenesis of T1D and providing another plausible explanation to the break in tolerance observed [100].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The hypothesis that diabetes could result from a 'normal' defence mechanism against beta cells that have accumulated changes and aberrations narrow the differences between tumor and autoimmune disease (see Outstanding questions). Both effective anti-tumor immunity and T1D would follow similar rules leading to recognition of self-modified peptides. Although the participation of mutations in T1D is unlikely, the fact remains that many gene variants can predispose to T1D. Polymorphisms of islet autoantigens have been reported to be immunogenic [77,78]. Intriguingly, T1D patients preferentially developed islet autoantibodies against the autologous variant of zinc transporter-8 [78,79].

Clinical cases reinforce the interdependence between tumor and diabetes. T1D patients tend to have lower risk of cancer, at least before insulin therapy. In two patients with repeated hypoglycaemia, surgical removal of their insulinoma revealed clinical signs of T1D with increased level of islet specific autoantibodies (Insulin, IA-2, GAD65 and ZnT8). Interestingly, the presence of insulitic lesions in the resected pancreas suggests priming anti-islet autoimmunity [80]. We speculate that this was the result of collateral damage of a well-intended response against the insulinoma. Similarly in case of myasthenic syndromes, where successful immunity against thymoma or small cell lung carcinoma can result respectively in myasthenia gravis and Lambert-Eaton myasthenic syndrome, autoimmunity has been confirmed against native self-proteins in the neuromuscular synapse [81,82]. Autoreactivity against PTM has hitherto not been assessed in these cases. Though, the link between islet autoimmunity and successful tumor immunotherapy became overwhelmingly clear recently, in studies using a check point blockade (inhibition of the

regulatory co-stimulatory molecule PD-1, e.g. by monoclonal antibody therapy with pembrolizumab or involumab) [83]. Removing the hand-break of the immune system released effective anti-tumor immunity in particular against mutated epitopes [84,85]. Vaccination studies using tumor antigens as immunotherapeutic strategy also may benefit from using mutated proteins rather than native tumor antigens, be it that the mutations may be patient or even metastasis specific[86]. These findings from effective cancer therapy may bear relevance to understanding natural immune regulation, as well as breaking immune tolerance to islet proteins in T1D. Perhaps modified islet autoantigens rather than native self-proteins act as primary targets leading to islet autoimmunity and beta cell destruction. Intriguingly, acute development of T1D ranks amongst the primary side effects of successful immunotherapy of cancer patients with anti-PD1 antibodies [87]. This last lesson from cancer also underscores the important role of PD-1 ligation in immune regulation in both progressive tumors and protection from T1D that may guide the way to novel immunotherapeutic intervention strategies [88].

Outstanding questions

- How do environmental factors and conditions affect antigen modification in T1D? Are these modified antigens a cause, consequence or epiphenomenon in the pathogenesis of T1D?
- Does intra-islet communication contribute to beta cell functionality in health and disease? Pancreatic islet have an architecture favoring beta-alpha cell interactions; the inter-endocrine dialog may provide insight in molecular mechanisms in homeostasis versus T1D pathology.
- Are mutations involved in T1D similar to effective antitumor immunity? Changes in either targeted autoantigens, T cell receptors, or autoreactive T cells may affect immunogenicity, immune regulation and microenvironment.
- Why is destructive autoimmunity in T1D limited to insulin-producing beta cells? Insulitis is driven by the presence of beta-cells.
- Why does the disease present in focal lesions in the pancreas? Does this point to beta cell or islet heterogeneity?
- Is T1D the cost of a well-intended response to stressed and dysfunctional tissue? The immune system is trained to detect and remove tissue featuring changes as induced by infection or carcinogenesis.

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CHAPTER 3.2

A roadmap of the generation of neoantigens as targets of the immune system in type 1 diabetes

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of the insulin-producing beta cells. Beta cell dysfunction caused by an inflammatory microenvironment is believed to trigger the peripheral activation of CD4 and CD8 autoreactive T cells. This review will compile post-transcriptional and post-translational modifications (PTM) involved in the generation of beta cell neoantigens and proposes a reconstruction of the sequence of events connecting environmental changes and autoimmunity.

Highlights

- Beta cells communicate with other endocrine cells and immune cells during the course of T1D.
- Beta cells contribute to their own demise by generating neoantigens targeted in T1D pathogenesis.
- Inflammation affects splicing, degradation and modification of proteins and peptides.
- PTM should be investigated as candidate targets of autoantibodies and CD8 T cells.

INTRODUCTION

Insulin producing beta cells are targeted by islet-infiltrating immune cells that recognize beta cell autoantigens in type 1 diabetes (T1D). Activated islet-autoreactive T cells occur in both circulation and insulitic lesions [1-3], but it remains poorly understood how these cells avoid thymic education, central and peripheral tolerance and immune regulation, and how they are primed. Improper presentation of self-antigens by antigen-presenting cells due to low-affinity native antigens may trigger these T cells to escape thymic deletion [4-6]. Besides failures in central tolerance, it is conceivable that beta cells are key-players in their own destruction by generating neoantigens avoiding central tolerance [7].

Accumulating evidence supports that local inflammation surrounding the islet of Langerhans alters the beta cell transcriptome and proteome, affecting the beta cell autoantigen repertoire [8]. In addition, beta cells are not just passive victims but actively participate in a dialogue with the immune compartment by locally releasing cytokines and chemokines upon insulitis [9-11]; the human islet architecture favors a direct cross talk between alpha and beta cells, suggesting a participation of intra-islet communication in balancing the islet micro-environment [12,13]. Here, we will make the case for discordances between the peripheral and thymic antigen repertoire to contribute to the lack of, or break in, peripheral tolerance by neoantigen-mediated beta cell destruction in T1D. We will also attempt to position genetic predisposition to disease, and link islet and macro-environment to the generation of neoantigens and loss or lack of immune tolerance. Finally, a connection between gut resident pathogens and the course of T1D in relation to neoantigens will be proposed [14].

GENE POLYMORPHISMS AND POST-TRANSCRIPTIONAL MODIFICATIONS IN AUTOIMMUNE DISEASE

Haplotypes at the human leukocyte antigen (HLA) loci are strongly associated with genetic risk for T1D development (in particular HLA-A2 and HLA-DQ). HLA molecules dictate the repertoire of peptides to be presented to the immune system and shape the adaptive immune system in the thymus. Genome wide association studies identified an additional 40 common polymorphisms

associated with T1D susceptibility [15]. Moreover, genetic variations or single nucleotide polymorphisms (SNPs) may affect regulatory elements or dictate RNA splicing. In T1D, allelic variation in the variable number of tandem repeats (VNTR) of the insulin gene were found to correlate with thymic insulin expression dictating the peripheral tolerance to insulin [4,16,17]. Also, SNPs in cytotoxic T lymphocyte-associated protein 4 (CTLA4) [18], expressed on the surface of activated CTLs and crucial for regulating peripheral T cell responses, are associated with a loss of self-tolerance. A SNP within the CTLA4 3'UTR led to an alteration in the ratio of CTLA4 mRNA splice isoforms of the *CTLA4* gene. This shifted splicing efficiency correlated with increased disease susceptibility. We recently discovered that the same SNP was associated with islet autoimmunity by affecting CTLA4 mRNA stability [19]. Similarly, rare deleterious variants in *PTPN22* in T1D patients with multiple-affected siblings were found to result in CD4 T cells hyporesponsive to antigen stimulation [20]. Rare SNPs in non-coding regions can alter regulatory elements such as promoter sequences or 3' untranslated regions (UTRs). This influences gene expression levels by interfering with miRNA regulation [21], a mechanism of post-transcriptional modification. In support of this, variation of functional miRNA-binding elements within the 3'-UTRs of T1D-associated genes (*CTLA4* and *IL10*) can have consequences for regulation of T1D risk genes [22].In addition to genetic variations, post-transcriptional modifications may contribute to autoimmune diseases by the generation of antigens escaping tolerance induction. The differential expression of IGRP variants between beta cells versus thymus was shown to result in the generation of islet neoantigens and the high immunogenicity against these splice products detected in both non-diabetic and type 1 diabetic subjects points to a lack of central tolerance [23]. Alternative splicing of IA-2 led to differential IA-2 expression in the pancreas versus the thymus which may underlie a permissive mechanism for the potential development of autoimmunity against IA-2 [24].

POST-TRANSLATIONAL MODIFICATIONS OF ISLET ANTIGENS IN TYPE 1 DIABETES

Post-translational modifications (PTM) increase the proteome and affect epitope binding properties to HLA class I and class II molecules, predisposing

to autoimmune diseases by changing the intrinsic structural and electrical properties of proteins or epitopes [25,26]. Polymorphic HLA-DQ molecules predisposing to risk for autoimmune diseases such as T1D favor certain peptide repertoires to be presented to the immune system and modifications of proteins and fragments may introduce peptide affinity, locking such antigenic peptides into the HLA groove [27] (Figure 1).

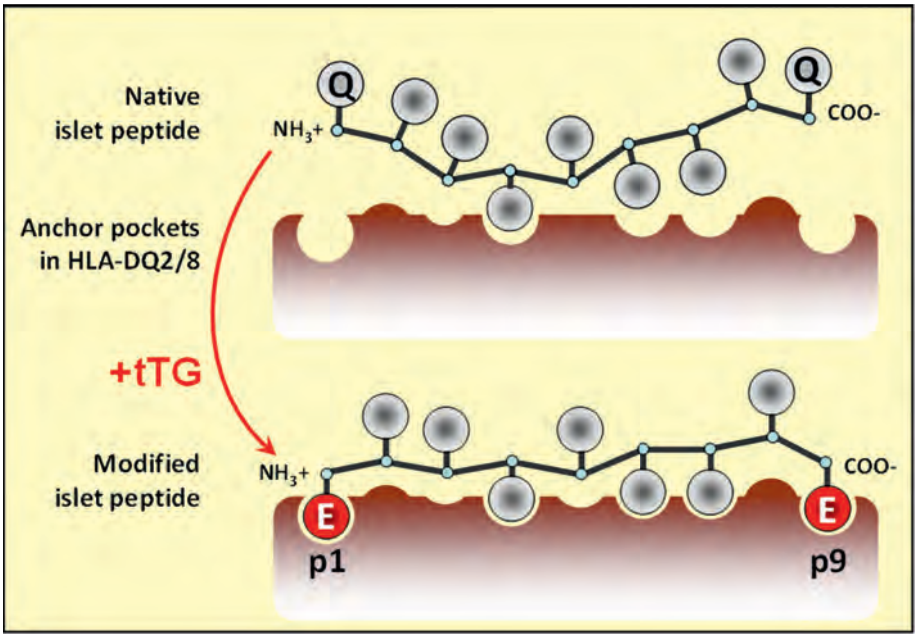

Figure 1: The key fitting the lock: deamidation of islet peptides promotes their binding to disease-predisposing HLA class II molecules. Peptide binding to HLA-DQ molecules involves anchor pockets 1, 4, 6, 7 and 9 of the peptide. Amino acids at position 1 and 9 of a binding peptide are crucial in defining peptide binding affinity to HLA-DQ8, whereas amino acids on positions 2, 3, 5 and 8 of the peptide epitope are engaged in recognition by T cell receptors (TCRs) (Top). Active tissue transglutaminase (tTG) can modify glutamines (Q) on positions p1 and p9 of a binding peptide by into glutamic acid (E), introducing a more favorable charge to the anchor pockets 1 and 9 (bottom), thereby strongly enhancing the binding affinity of this peptide to HLA-DQ8.

Animal models of autoimmune diabetes showed that PTMs create new autoantigens recognized by diabetogenic T cells [28]. The first evidence of PTM in T1D in humans involved chemical modification in the form of a disulfide bond between adjacent cysteine residues on the A chain of human insulin as a target of T cells [29]. Enzymes can create another range of modified antigenic peptides. Tissue transglutaminase (tTG) is an enzyme responsible for modifying gluten

and gliadin into allergens in coeliac disease by deamidation of glutamine into glutamate. This enzyme is also present in relative high levels in pancreatic islets [www.proteinatlas.org], but becomes active in the context of inflammation [30]. Indeed, deamidated proinsulin was recently demonstrated in inflamed human islets of Langerhans [30]. We recently showed that active tTG deamidates proinsulin, thereby promoting binding affinity to high-risk HLA-DQ molecules [34]. T cell responses directed against deamidated proinsulin were observed in the majority of the newly diagnosed T1D patients, compared to less than half of the patients responding to native proinsulin. Perhaps surprising, deamidated islet epitopes could also be isolated from high-risk HLA class II of dendritic cells pulsed with native islet antigens, implying that dendritic cells, too, are able to post-translationally modify, process and present deamidated neoepitopes and turn these into potent autoantigens in T1D [33]. The increased tTG activity in islet cells and dendritic cells suggests that tTG is responsible for this inflammation-induced modification. The conversion of (protein-bound) arginine into the non-standard amino acid citrulline by peptidylarginine deiminases (PADs) is interesting in the context of autoimmunity since PADs have not been detected in the thymus [31], possibly excluding T cell education toward citrullinated peptides. Citrullination of GAD65 peptides enhances the binding to HLA-DR4; these peptide-HLA complexes were recognized by autoreactive CD4 T cells isolated from T1D patients [25]. Also citrullinated glucose-regulated protein 78 was identified as a modified autoantigen in murine beta cells and was a target for autoreactive T cells in mice, indicating that PTM of islets autoantigens is directly involved in beta cell destruction [32]. Recently, a new class of modification wherein fusion peptides from non-contiguous peptide fragments generate novel epitopes has been reported to be involved in T1D [2]. In this study diabetogenic CD4 T cell clones isolated from non-obese diabetic mice recognized epitopes consisting of proinsulin peptides covalently crosslinked to other peptides present in insulin secretory granules. This peptide fusion, thought to be formed through a transpeptidation reaction, led to the creation of hybrid insulin peptides that were detectable in beta cells. CD4 T cell lines from the residual pancreatic islets of two type 1 diabetic pancreas donors also recognize such hybrid peptides [2]. These types of neoantigen generation may explain how immune tolerance is broken in T1D (Figure 2).

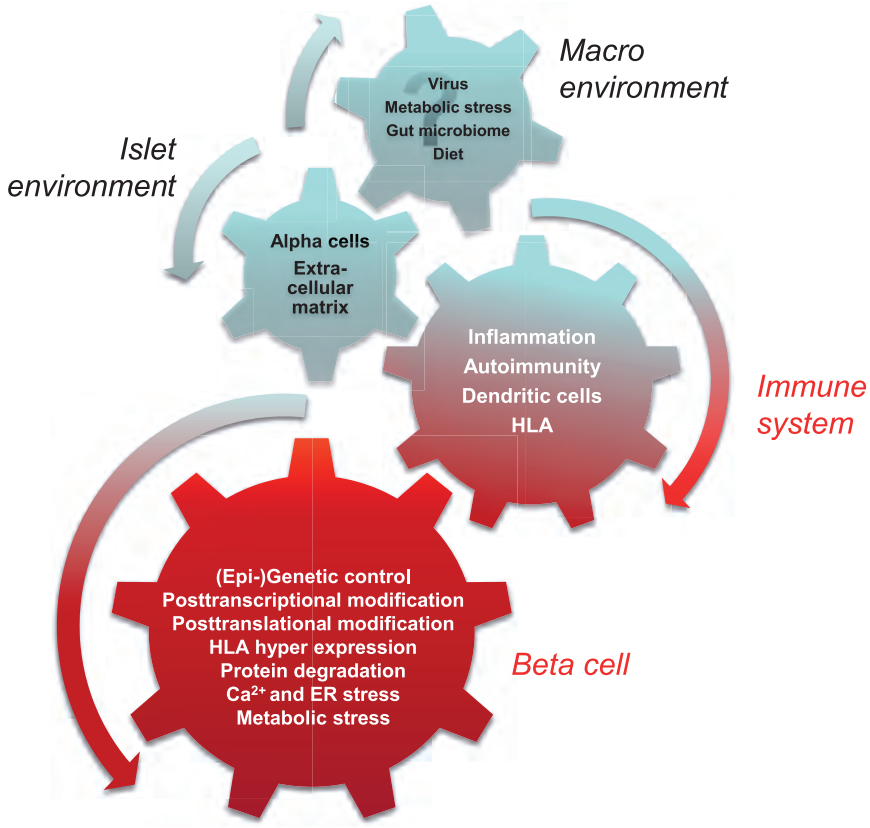

FIGURE 2. A roadmap to protein modifications in the development of T1D. Environmental changes due to infections by pathogens or change in diet may affect the immune system. Cytokines and chemokines secreted by both innate immune cells and adaptive immune cells may interfere with the communication between beta cells and other pancreatic cells and disturb post transcriptional and post translational fidelity in beta cells. Such processes may contribute to the generation of modified autoantigens. Of note, environmental changes would also contribute to the generation of autoantigens by modification of native proteins or peptides taken up and modified by dendritic cells.

An important function of PTM is regulation of protein localization. Upon inflammation-induced ER stress, the palmitoylation cycle of GAD65 is disrupted [33]. This results in an aberrant endomembrane distribution and accumulation of palmitoylated GAD65 in Golgi membranes which accordingly triggers autoimmunity, presumably by increased uptake and processing by APCs.

Although a role of autoantibodies in T1D pathology is still unproven, insulin autoantibodies (IAA) remain the earliest marker of beta cell autoimmunity. Interestingly, among the rare patients being IAA- negative, more than 30%

were tested positive for autoantibodies against modified insulin [34]. The same group showed the presence of autoantibodies against modified collagen [35], suggesting that autoantibodies against modified islet antigens can add significant value for (early) diagnosis of T1D. Yet, humoral autoimmune responses to neoantigens have largely been ignored. It is not inconceivable that autoantibodies to native self-proteins only represent the tip of the iceberg. This assumption is corroborated by the situation in other inflammatory diseases, such as coeliac disease and rheumatoid arthritis.

THE ROLE OF INFLAMMATORY CYTOKINES IN THE GENERATION OF ISLET NEOANTIGENS

During acute insulitis, the combined effect of proinflammatory cytokines and chemoattractants on beta cells leads to hyperexpression of HLA class I, triggering beta cell exposure to immune cells and the selective infiltration of autoreactive $CD8^+$ T cells [1]. More than just recruiting immune cells to the inflammation site, inflammatory signals interfere with cellular processes during conversion of genetic information into proteins. At the RNA level, IFN-γ, IL1β combined treatment led to profound changes in beta cell gene expression, including genes involved in the splicing machinery [36,37]. As a consequence, transcriptome analysis of purified human islets maintained in proinflammatory cytokines has shown that over 30% of the genes expressed in human islets undergo alternative splicing, providing evidence for the generation of beta cell neo-autoantigen generation during insulitis [8].

Inflammatory cytokines perturb the intracellular calcium level, affecting the ER homeostatic balance and activating Ca^{2+} dependent enzymes involved in post-translational modification (e.g., tTG and PAD [38,39]). Recently, we have shown that supernatant of an activated GAD65-specific CD4 T cell clone induced post-translational modifications of human islet antigens and in particular, deamidated peptides derived from PPI were [30] a prototype target for autoreactive T cells in T1D patients [40,41]. Although the Unfolded Protein Response triggered by ER stress is highly involved in PTM modification in T1D [42], a prolonged exposure to metabolic or inflammatory stress may also promote additional coping mechanisms, including initiation of recycling programs by vesicle formation [43]. In synoviocytes and monocytes isolated

from RA patients, m-TOR activation by Rapamycin was shown to activate PAD activity in autophagosomes and was proposed as mechanism for citrullination [43].

The local inflammation is an important component of beta cell destruction and in the generation of PTM modified epitopes. However, the identity of pro-inflammatory cytokines in the vicinity of the inflamed beta cells appears to determine the type and the intensity of beta cell stress [44,45]. Unfortunately, studying the interplay between the immune compartment and the islet compartment is challenging and our knowledge from the role of cytokines in T1D, largely derived from studies in rodent or cell lines with excessive un(patho) physiological cytokine concentrations, lacks consistency. For example, the mediators themselves (e.g., IFN-γ or TNF alpha) seem to both promote and prevent beta cell destruction [46]. But more importantly, in humans the situation might not be necessarily the same! Our view of the role of cytokines in human T1D is limited and *"in situ"* investigations, where biopsies from living subjects are analysed, are rare but suggest that the amount of cytokines secreted by infiltrating immune cells within T1D islets might have been largely overestimated [47].

Inflammation also alters processing of the HLA class I binding peptide repertoire. The subunits b1, b2, b5 from the 20S proteasome catalytic domain are replaced under inflammation by their inflammatory counterparts. Hence, the immunoproteasome formed may reshape the peptide repertoire and the potential CTL response [48] by different proteolytic activity, affecting processes like transpeptidation [49,50] that could contribute to ligandome diversity. Of note, CD8 T cell autoimmunity to modified proteins has not yet been investigated in any detail in humans, which is remarkable, given the large body of recent evidence of CD4 T cell autoimmunity against PTM products.

THE INFLUENCE OF THE ENVIRONMENT

Other pancreatic endocrine or exocrine cells have been largely ignored in the selective beta cell destruction process. Studies are supporting a guardian-type role of alpha cells in particular, under stress stimulation by GLP-1 secretion [51,52], but consequences of GLP-1 stimulation or other incretins on the beta cell proteome still remain to be investigated. Undoubtedly, the islet micro-

environment is relevant [53]. We recently discussed the inverse relationship between the tumour microenvironment and islets during insulitis [54].
The macro-environment also contributes to development of T1D, and its effect on autoantigenesis should be considered. The notion that approximately 50% of identical twins are discordant for T1D demonstrates that T1D is not an inherited genetic disease and is largely influenced by environmental factors, possibly acting in concert with genetic risk factors [55]. The gut and the pancreas are linked through a system in which gut-derived immune and metabolic signals have the potential to induce effects in the pancreas. The gut microbiome influences host metabolism and immunity and might play a role in T1D pathogenesis. Although profound changes in host gene expression and proteome were observed following enterovirus infection, the link between virus infection and generation of beta cell neoantigens remains unestablished. Similarly, recent microbiome analysis pointed to a correlation between bacteria in Finnish and Estonian infants and autoimmune diseases [56], indicating the possible implication of a "gut storm" in T1D pathology [57]. Although a direct link with the generation of modified antigens in T1D was not tested, studies in coeliac disease and rheumatoid arthritis revealed a direct effect of microbial tTG from *Streptoverticillium* and PAD from *Porphyromonas gingivalis* enzymes on PTM modification of host substrates [58,59]. It is conceivable that such processes may also occur in T1D.
The importance of the gut in the pathology of T1D is also illustrated by the role of diet changes in autoimmune development. Gluten free diet decreases intestinal inflammation and prevents CD development [60,61]. Similarly, such nutritional intervention may impact the gut microbiome and delay incidence of T1D as demonstrated in rodents [62]. In patients with both CD and T1D, a gluten-free diet improves glycaemic control in some studies, but not consistently [63]. Children with anti-islet cell autoimmunity presented an *Akkermansia*-dominated gut microbiome resulting in higher levels of butyrate production with subsequent protective effects on development of T1D [64]. A relation with PTM is yet to be investigated.
The question remains whether PTM provokes or results from islet autoimmunity? Does the surrounding islet micro-environment or macro-environment generate PTM of islet antigens priming naïve T cells? Intriguingly, be it inconclusive, T cell lines were generated against modified, but not native proinsulin [65]. Once activated, these T cells cross-reacted with native proinsulin. This could

imply that PTM epitopes act as initial targets, leading to the loss of tolerance in autoreactive T cells in T1D patients.

CONCLUDING REMARKS

Modified islet epitopes are newly appreciated actors in T1D pathogenesis. A better understanding of the processes and micro- and macro-environmental factors contributing to the generation of protein modifications to which immune tolerance is lacking or lost may prove essential for prevention or intervention of type 1 diabetes. Appreciating the role of beta cells in their own fate, in dialogue with other islets cells, the immune system and the gut microbiome, has brought new dimensions to deciphering changes in immune homeostasis in the pancreas during insulitis. This is critical to define the terms of loss of islet autoimmunity and the restoration of immune tolerance.

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CHAPTER 4

Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes

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ABSTRACT

Identification of epitopes that are recognized by diabetogenic T cells and cause selective beta cell destruction in type 1 diabetes (T1D) has focused on peptides originating from native beta cell proteins. Translational errors represent a major potential source of antigenic peptides to which central immune tolerance is lacking[1, 2]. Here, we describe an alternative open reading frame within human insulin mRNA encoding a highly immunogenic polypeptide that is targeted by T cells in T1D patients. We show that cytotoxic T cells directed against the N-terminal peptide of this nonconventional product are present in the circulation of individuals diagnosed with T1D, and provide direct evidence that such $CD8^+$ T cells are capable of killing human beta cells and thereby may be diabetogenic. This study reveals a new source of nonconventional polypeptides that act as self-epitopes in clinical autoimmune disease.

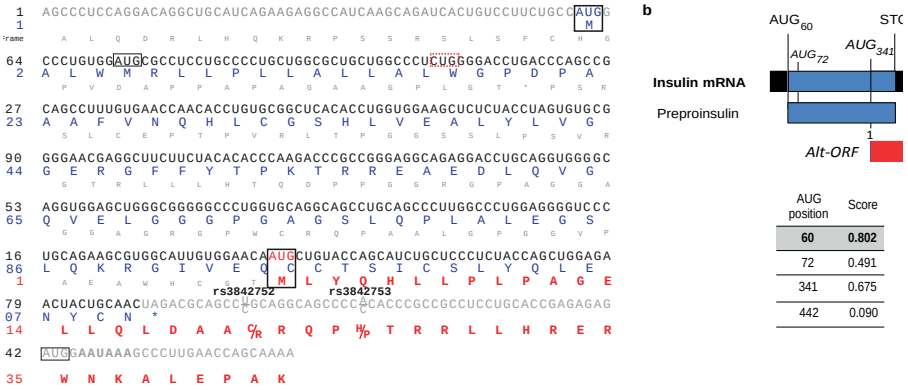
Islet-reactive CD8+ T cells selectively and progressively destroy the insulin-producing beta cells in type 1 diabetes (T1D)[3-6]. Metabolic or inflammatory stress in the vicinity of the beta cells may contribute to the generation of neoantigens to which central immune tolerance is absent, thereby triggering autoimmunity [7, 8]. For example, larger numbers of splicing events are detected in human beta cells maintained *in vitro* in the presence of proinflammatory cytokines, mimicking the pathophysiological conditions of T1D[9, 10]. In addition, CD4+ T cells that recognize deamidated autoantigens[11-13] or fusion epitopes[14] have been detected in individuals with T1D. Inflammatory stress may perturb the cellular equilibrium and affect high-fidelity transcriptional and translational processes during conversion of the genetic information into proteins[15, 16]. In tumors, uncontrolled cell proliferation correlates with enhanced translation and accumulation of aberrant translation products. Such so-called defective ribosomal products (DRiPs)[17-19], arising from translation of normally untranslated regions (UTR), ribosomal frame-shifting or alternativeinitiation of translation, generate a unique class of tumor-associated antigens that are selectively expressed by malignant cells[20, 21].

Human pancreatic beta cells are insulin factories dedicated to the maintenance of glucose homeostasis; insulin, stored in secretory granules, represents 10-15% of the protein content of these cells[22, 23]. Upon glucose challenge, insulin molecules are released into the circulation by exocytosis and insulin mRNA is rapidly translated by polysomes to increase insulin biosynthesis. Studies of samples from humans with T1D and mouse models of the disease indicate that native insulin and its precursors act as primary autoantigens[24-26], and fragments of signal peptide of the preproinsulin (PPI) were identified as main targets of cytotoxic islet-autoreactive CD8+ T cells in human T1D[27]. In a process similar to the emergence of DRiPs during tumor development, the high demand for insulin may lead to the generation of aberrant insulin polypeptides by impaired translation fidelity, rendering beta cells immunogenic. These errors may yield antigens that trigger or accelerate islet autoimmunity in T1D, particularly as a result of stresses that, for example, may be evoked by viral infection[28], reduced pancreas size[29] or inflammation[30]. Here, we investigated whether beta cells produce aberrant translation products that can lead to the generation of diabetogenic epitopes.

Leaky ribosome scanning for translation initiation at a downstream AUG can generate out-of-frame translation products[31]. Within the human insulin

mRNA, two putative downstream translation initiation sites with strong Kozak consensus sequences are located at positions 72 and 341. Although translation initiation on the AUG at position 72 (which is in frame with the canonical AUG of PPI) would produce a truncated isoform of PPI, initiation on the AUG at position 341 would generate an alternative polypeptide in a +2 reading frame that does not share any sequence identity with the canonical translation product of the insulin gene (Fig. 1a,b). The absence of a stop codon in this frame would lead to translation into the poly(A) tail. To validate that translation initiation can occur at this particular AUG, green fluorescent protein (GFP) fusion constructs were generated, in which the GFP coding sequence was fused in frame with the bona-fide PPI AUG_{60} (insulin(INS)-GFP) or with the alternative AUG at position 341 (INS-DRiP-GFP). As a control, a third construct was generated, in which the alternative reading frame was cloned immediately after the CMV promoter (DRiP-GFP) (Fig. 2a). Following transfection in HEK 293T cells, in order to achieve high protein expression, both INS-GFP and INS-DRiP-GFP constructs led to expression of GFP fusion proteins migrating at 40 kDa and 33 kDa, respectively. The control construct, DRiP-GFP, confirmed the molecular weight of the unconventional product (Fig. 2b). As expected, cells transfected with INS-DRiP-GFP also coexpress proinsulin as assessed by western blot (Fig. 2c) and immunohistochemistry (Fig. 2d).

To determine the effect of endoplasmic reticulum stress (ER stress) on translation initiation, cells transfected with INS-DRiP-GFP were stimulated with the ER stress-inducers thapsigargin (TG) or tunicamycin (TM). The expression of DRiP polypeptide was increased by TG but not TM treatment as quantified both by western blot (Fig. 2e, upper) and flow cytometry analysis (Fig. 2e, lower). Altogether, these data indicate that the production of the INS-DRiP polypeptide may result from calcium depletion in the ER and the subsequent increase in levels of cytoplasmc calcium, rather than the classical ER stress caused by assumulation of misfolded proteins as induced N-linked glycosylation inhibition (TM)[32].



AUG position	Score
60	**0.802**
72	0.491
341	0.675
442	0.090

Figure 1: Schematic representation of open reading frames (ORFs) found in the human insulin mRNA. (a) Full-length insulin mRNA with the bona fide PPI ORF (black uppercase letters), 5′ and 3′ UTRs (grey uppercase letters) and the poly(A) signal sequence (bold grey letters) are shown. The PPI amino acid sequence is shown in blue font, the amino acid sequence of the +2 reading frame is shown in small, light grey font and the amino acid sequence of the alternative open reading frame (altORF) INS-DRiP is depicted below the mRNA sequence in bold red. . All AUG codons within the mRNA are framed with a black box, and those used as translation initiation site are indicated with colors corresponding to the resulting amino acid sequence. The * indicates the stop codon in the given amino acid sequence. The putative nonAUG (CUG) start site upstream of the DRiP sequence is framed with red dashed line. The 3′-UTR SNPs are annotated and both polymorphisms are depicted, as are the potentially affected amino acids in the nonconventional polypeptide. (b) Left, schematic representation of full-length human insulin mRNA. The 5′- and 3′-UTR are depicted in black and the insulin-encoding ORF starting at AUG_{60} in blue. Alternative translation initiation sites are shown in italic and the poly(A) tail is indicated in bold. The altORF encoding the INS-DRiP polypeptide is shown in red, and the first amino acid (1) and the last amino acid preceding the poly(A) tail (43) are depicted. Right, translation initiation scores of every AUG within the insulin mRNA sequence as predicted by NetStart 1.0 prediction server. Prediction scores greater than 0.5 are considered probable translation start codons.

On the basis of the insulin sequence, translation of this neopolypeptide continues beyond the original PPI stop codon and leads to translation of two single nucleotide polymorphisms (SNPs) (rs3842752 and rs3842753) that are in strong linkage disequilibrium and are associated with development of T1D[33]. Although these SNPs have been judged to be functionally irrelevant because of their location within the *INS* 3′UTR[34], in this scenario, the SNPs rs3842752 and rs3842753 may generate four different polypeptide variants of the INS-DRiP polypeptide containing, respectively, cysteine-to-arginine (C-to-R) and histidine-to-proline (H-to-P) substitutions that may act as neoantigens in T1D (Fig. 3a). To test the immunogenicity of the INS-DRiP variants, T cell proliferation assays were performed on freshly isolated peripheral blood mononuclear cells (PBMCs) of juveniles with T1D patients for detection of islet autoimmunity (Supplementary Table 1 and Fig. 3b). Proliferative responses were detected in the majority of T1D cases that strongly correlated in reactivity between

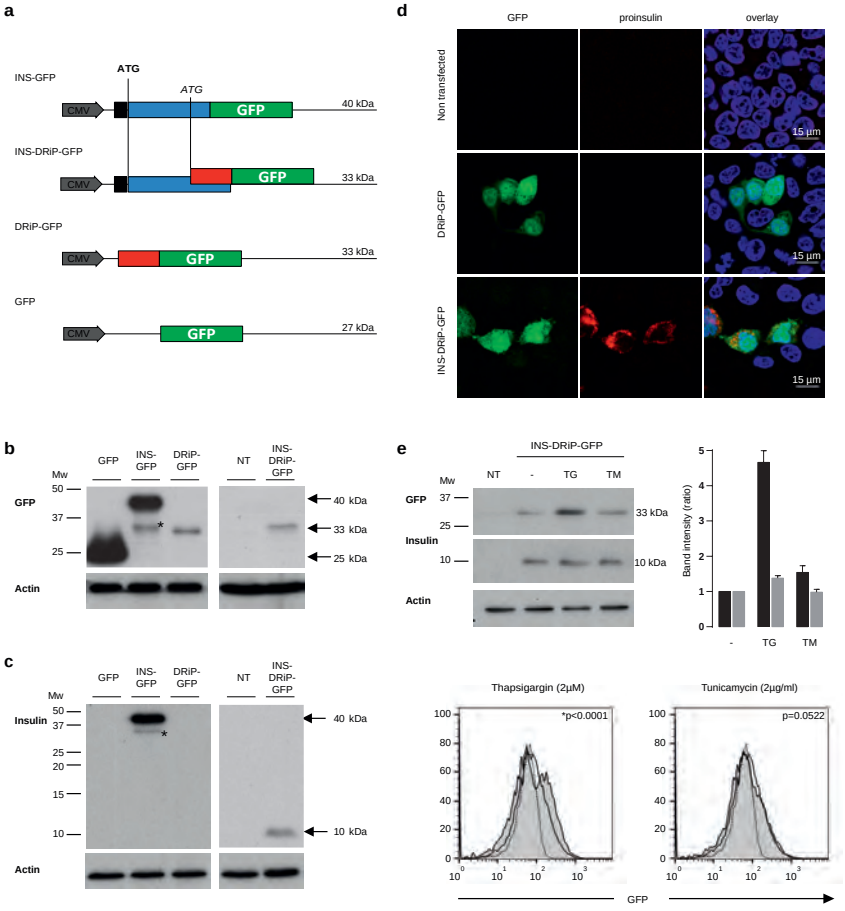


Figure 2: The human insulin mRNA harbors an alternative open reading frame. (a) Schematic representation of the GFP fusion constructs INS-GFP, INS-DRiP-GFP, DRiP-GFP and the normal GFP construct. The canonical PPI AUG is shown in bold and AUG_{341} is shown in italic. The expected size of the GFP protein and the fusion variants are depicted on the right. The sequences encoding preproinsulin and the INS-DRiP are depicted in blue and red, respectively. The sequence encoding GFP is shown in green. The 5'-UTR is shown in black. (b) Western blot analysis of 293T cell lysates transfected with GFP, INS-GFP and DRiP-GFP constructs (left panel) and 293T cell lysates either nontransfected (NT) or transfected with the INS-DRiP-GFP construct (right panel). Western blot analysis was performed 48 h post transfection and analyzed with an anti-GFP antibody (top panels) or anti-actin antibody used as loading control (lower panels). (c) Western blot analysis using anti-insulin antibody (top panels) or anti-actin antibody (lower panels) of the previously described transfected cells. * indicates an INS-GFP degradation product visible by anti-GFP and anti-insulin-staining. (d) Confocal images of 293T cells that were transiently transfected with DRiP-GFP and INS-DRiP-GFP constructs. The left panels show the GFP fluorescence of the GFP fusion variants, the middle panels show the expression of proinsulin in the transfected cells and the right panels show the overlay with DNA staining as seen by Hoechst staining. (e) Analysis of the effect of ER stress on translation initiation. INS-DRiP-GFP transfected cells were stimulated 24 h post transfection with Thapsigargin (TG 2 µM) or Tunicamycin (TM 2µg/ml) for 6 h. DRiP and proinsulin expression were evaluated after western blot analysis using anti-GFP and anti-insulin antibodies, respectively. Actin expression was used as loading control (left panel). Quantitative analyses

of the effect of stress inducers on protein translation was determined (i) by densitometry analyses of the GFP (black bars) and insulin bands (grey bars) and shown as induction ratio ± s.d. where the intensity of the nontreated cells was used as reference (right panel) and (ii) by comparing GFP fluorescence upon stress induction by FACS. Nontransfected cells were used as control (dashed lines in both histograms). GFP expression of the INS-DRiP-GFP transfected cells in nontreated and treated conditions are depicted with white-filled and grey-filled histograms, respectively. Statistical analysis of the GFP-positive population in **e** was performed using an unpaired two-tailed Student's t-test (n=3 from 2 independent transfection experiments).

the 'susceptible' (R-P) and 'protective' (C-H) INS-DRiP variant, suggesting that the SNP region itself did not contribute to immunogenicity and underscoring the lack of presentation of the SNP by HLA-DQ (Fig. 3b, left). Intriguingly, the few individuals carrying genetic risk variants associated with protection from T1D (HLA-DQ6.2 or INS SNP C-H) did not respond to INS-DRiP polypeptides, supporting the hypothesis that DQ6.2 and INS SNP C-H contribute to central immune tolerance in thymic education (Fig. 3b right). Strong T cell responses to INS-DRiP were detected in individuals with increased genetic risk for T1D (those heterozygous for HLA-DQ2 and HLA-DQ8 (HLA-DQ2/8); i.e., HLA-DQ8trans (formed by the α-chain of HLA DQ2 (DQA1*05:01) and the β-chain of HLA-DQ2 (DQB1*03:02))or HLA-DQ2trans(formed by the α-chain of HLA-DQ8 (DQA1*03:01) and the β-chain of HLA-DQ2 (DQB1*02:01)) (Fig. 3c). Of note, the T cell response to INS-DRIP was similar in amplitude in comparison to the other known antigen targets in T1D (Supplementary Fig. 1a-c).

Next, we interrogated whether dendritic cells (DC), which are uniquely capable of priming the immune system, were able to process and present peptides from the INS-DRiP polypeptide. Monocyte-derived, immature DC generated from DQ2- or DQ8-homozygous, or DQ2/8-heterozygous donors were pulsed with the INS-DRiP variants. After 24 h of culture, the DC HLA-DQ ligandome was determined by mass spectrometry, revealing a short peptide fragment of 9 amino acids from the N-terminus of the INS-DRiP polypeptide as a single candidate epitope. A full overlap of the experimental fragmentation profile of the identified peptide and its synthetic counterpart confirmed proper MS identification (Fig. 3d). Cell-free HLA-DQ binding studies confirmed a strong binding affinity of the 9-mer peptide to HLA-DQ8cis and HLA-DQ8trans molecules but not to HLA-DQ2cis or HLA-DQ2trans molecules (Fig. 3e). These data show that the INS-DRiP peptide fragment is naturally processed by DCs and is preferentially presented by T1D highest-risk HLA-DQ8trans, which is expressed in DQ2/8 heterozygous individuals. Also, monitoring the immune response to INS-DRiP$_{1\text{-}9}$ by interferon (IFN)-y and GrzB Elispot indicates that the

peptide-stimulated production of granzyme B occurs exclusively in individuals with T1D (Supplementary table 1 and Supplementary Materials). Together, these results indicate that the N-terminus of the INS-DRiP polypeptide is particularly immunogenic and suggests that a cytotoxic T lymphocyte (CTL) response occurs in T1D.

a

SNP	Allele	Frequency	Genotype	Frequency
rs3842752	A	0.033 (3)	A/A	0 (0)
	G	0.967 (89)	A/G	0.065 (3)
			G/G	0.935 (43)
rs3842753	T	0.109 (10)	T/T	0 (0)
	G	0.891 (82)	T/G	0.217 (10)
			G/G	0.783 (36)

Haplotype	Frequency	Phenotype	Frequency
A-T	0.033 (3)	C-H/C-H	0 (0)
A-G	0 (0)	C-H/R-H	0 (0)
G-T	0.076 (7)	C-H/R-P	0.065 (3)
G-G	0.891 (82)	R-H/R-H	0 (0)
		R-H/R-P	0.152 (7)
		R-P/R-P	0.783 (36)

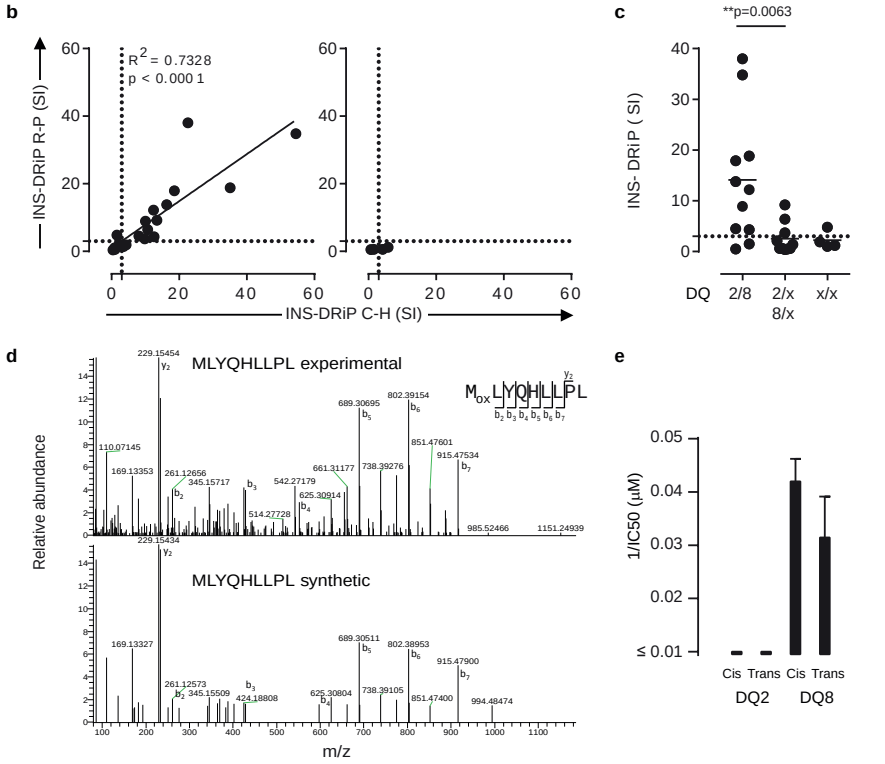


Figure 3: Immunogenicity of insulin-derived, nonconventional polypeptide. (a) Frequencies of allele, genotype, haplotype and phenotype of the T1D patient panel used in this study. Individual genotypes and phenotypes are depicted in **supplementary table 1**. The allele A or G in SNP rs3842752, result in a transcript with U or C leading toward expression of cysteine (C)- or arginine (R)-containing INS-DRiP, respectively; the allele T or G in SNP rs3842753 , result in a transcript with A or C leading toward expression of histidine (H)- or proline (P)-containing INS-DRiP, respectively. (b) Lymphocyte proliferation against the recombinant INS-DRiP variants (C-H and R-P) in PBMCs from fresh blood of individuals with T1D who are homozygous for the susceptible INS-SNP phenotype R-P/R-P (left) or heterozygous with the protective phenotype HLA-DQ6.2 or INS-SNP C-H (right). A significant correlation between immune

responses to the INS-DRiP R-P and C-H was determined by two-tailed Pearson correlation test (P<0.0001). (c) Proliferative responses against the recombinant INS-DRiP R-P polypeptide of PBMCs isolated from individuals carrying the high risk HLA-DQ2/8 genotype (DQ2/DQ8) in comparison to individuals carrying DQ2/x or DQ8/x and individuals negative for both DQ variants (DQx/DQx), where x represents any DQ molecule other than DQ2 or DQ8. The P value has been calculated by a Mann-Whitney t-test. (d) Mass spectrometry analysis of the MLYQHLLPL peptide eluted from HLA-DQ2/8 expressed on DCs pulsed with recombinant INS-DRIP polypeptide (tandem mass spectrum, upper panel) and the spectrum of the synthetic INS-DRiP$_{1-9}$ peptide (lower panel). (e) Binding validation of INS-DRiP$_{1-9}$ peptide to the T1D high-risk DQ2cis, DQ2trans, DQ8cis and DQ8trans molecules as shown by the experimentally determined IC_{50} values. Data represent mean ± s.e.m. (n=3 independent binding experiments).

The INS-DRiP polypeptide was screened for potential human leukocyte antigen (HLA)-class-I-binding epitopes. The INS-DRiP sequence was analyzed by three major histocompatibility complex (MHC) class I epitope prediction algorithms: NetMHC 3.4, SYFPEITHI and BIMAS. One strong HLA-A2-binding peptide was identified that was identical to INS-DRiP$_{1-9}$ eluted from HLA-DQ8trans (Supplementary Table 2a). The INS-DRiP$_{1-9}$ epitope MLYQHLLPL was confirmed to bind with high affinity to HLA-A2, which is the most prevalent HLA variant within the T1D population (Supplementary Table 2b and Supplementary Materials)[35, 36].

We next assessed the clinical relevance of the INS-DRiP$_{1-9}$ epitope by exploring the presence of specific CD8$^+$ T cells in peripheral blood samples of T1D HLA-A2$^+$ individuals with T1D and in HLA- and age-matched healthy donors (detailed in Supplementary Table 3)[37]. Significantly higher levels of INS-DRiP$_{1-9}$-specific CD8$^+$ T cells were detected in individuals with T1D as compared to healthy donors, whereas antiviral CD8$^+$ T cell frequencies were similar between the two groups (Fig. 4a). Phenotypical analysis of INS-DRiP$_{1-9}$-Tm$^+$ CD8$^+$ T cells showed more cells with the effector phenotype (CCR7$^-$/CD45RA$^-$) in donors with T1D than in healthy donors (Supplementary Fig. 2).

To further characterize these T cells, INS-DRiP$_{1-9}$-specific T cell clones were generated from PBMCs of individuals with T1D patients by dual HLA tetramer staining, and their cytotoxic properties were determined on peptide loaded HLA-A2$^+$ JY cells (Fig. 4b,c and supplementary Fig. 3). Of note, these DRiP-specific CTL clones were generated from an individual with long-term diabetes (clone #1) as well as an individual with new-onset diabetes (clone #2); this indicates that such T cells are participating at different stages of disease progression. Complete lysis of peptide-pulsed target cells was detected, whereas target cells loaded with an irrelevant peptide remained unaffected (Fig. 4c). Peptide-specific T cell activation resulted in a significant increase in the secretion of

IFNγ and tumor necrosis factor (TNF)-α and, to a lesser extent, macrophage inflammatory protein (MIP)-1β when compared to unstimulated T cells (Fig. 4d). We next examined the cytotoxic potential of the INS-DRiP$_{1-9}$-specific CTL on human islet cells expressing HLA-A2. In order to specifically investigate beta cell death, dispersed primary human islets were transduced by a lentivirus containing the beta cell-specific viability reporter (i.e., a short-half-life luciferase reporter gene under the control of the human insulin promoter, HIP-LUC2CP) as previously described[38]. Following LV-HIP-LUC2CP transduction, human pancreatic islet cells were incubated with CTLs specific for INS-DRiP$_{1-9}$, CMVpp65 or PPI$_{15-24}$, and cytolysis was determined by measuring reduction of luciferase activity. PPI$_{15-24}$-specific and INS-DRiP$_{1-9}$-specific CTLs significantly reduced beta cell survival (Fig. 4e, left), whereas beta cell survival was unaffected by CMV-specific CTLs[38]. Together, beta cell destruction by the INS-DRiP$_{1-9}$-directed CTLs confirmed that the DRiP$_{1-9}$ epitope is naturally generated, processed and presented on the cellular surface on human beta cells. The partial destruction of beta cell by INS-DRiP$_{1-9}$ specific CTL is in tune with the error hypothesis and points to beta cell heterogeneity and immunogenicity; this is perhaps due to differential sensitivity to stress factors. To test whether inflammation increases beta cell susceptibility to cytolysis by INS-DRiP$_{1-9}$-specific T cells, beta cells were preconditioned with medium containing high glucose combined with proinflammatory cytokines IL-1β and IFNγ to mimic T1D pathology (Fig. 4e, right). As anticipated from our results regarding the effect of ER stress on DRiP translation (Fig. 2f), these inflammatory conditions further increased beta cell death in the presence of INS-DRiP$_{1-9}$-specific CTLs (Fig. 4f). INS-DRiP$_{1-9}$ specific CTL cocultured with human islets consistently secreted the effector cytokines MIP-1β, IFNγ and TNF when recognizing the INS-DRiP epitope presented by HLA-A2, corroborating specific stimulation and activation of the CD8$^+$ T cells by islets cells (Fig. 4g).

We present the first evidence of naturally processed and presented epitope derived from nonconventional islet proteins leading to the destruction of human beta cells by cytotoxic CD8$^+$ T cells. We identified an immunogenic polypeptide translated from the insulin mRNA that is capable of triggering T cell proliferation in PBMCs from individuals with the highest odds ratio for T1D. In addition, we demonstrate that the epitope, presented by HLA-A2 as well as the highest-T1D-risk HLA-DQ8trans molecules expressed on DQ2/8 heterozygous DCs, is implicated in T1D pathogenesis as patient-derived

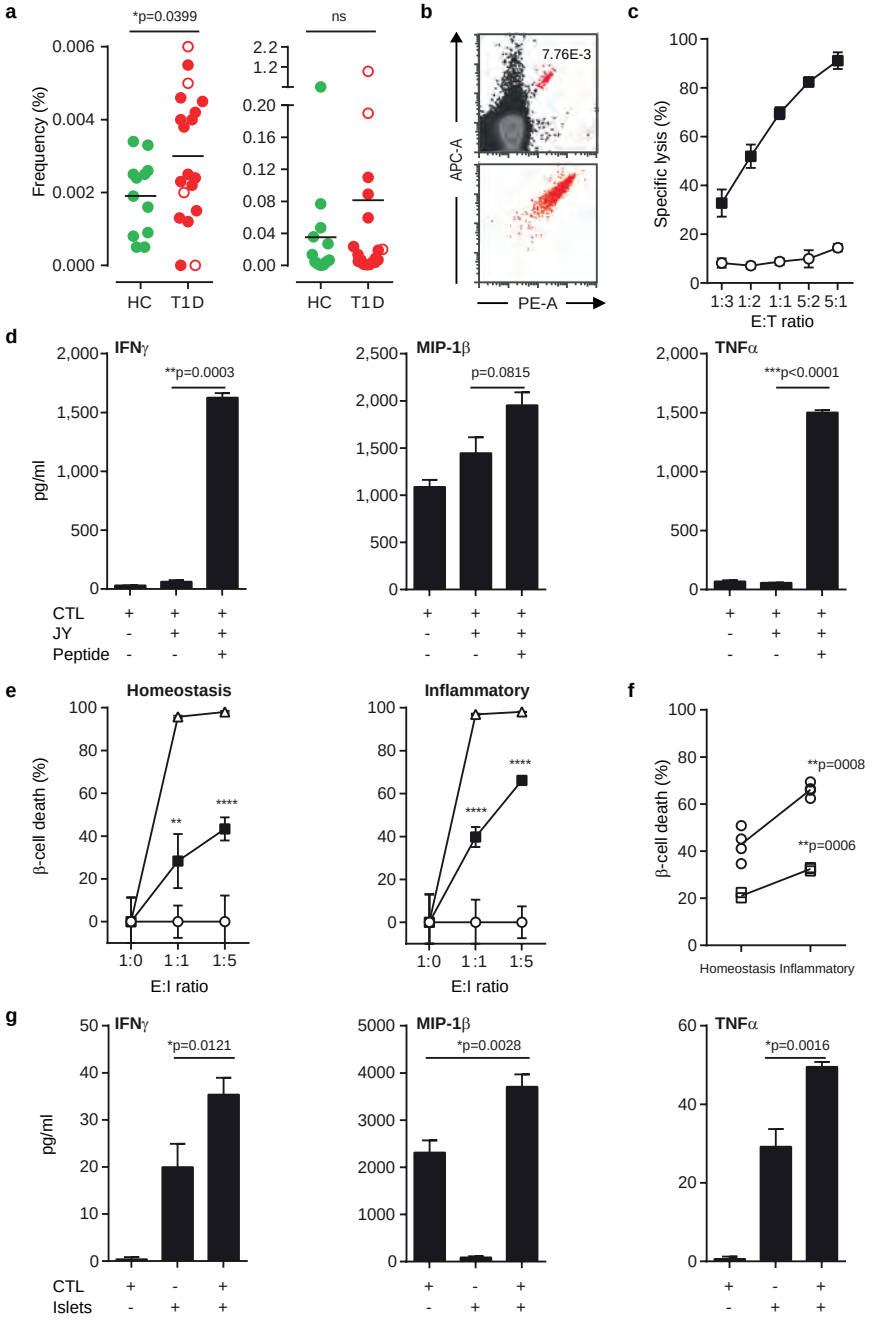


Figure 4: INS-DRIP$_{1-9}$-specific CTLs kill human beta cells *in vitro*. (a) Frequency of INS-DRiP$_{1-9}$-specific CTLs (left panel) and virus-specific CTLs (EBV/LMP2, measles/H250 and CMV/pp65) (right panel) detected in the circulation of individuals with T1D (red, filled circles), individuals with new-onset T1D (red, open circles) and matched healthy control subjects (green, filled circles). Statistical analyses of the

two groups were performed using GraphPad; an unpaired t-test with Welch correction was applied. (b) Generation of INS-DRiP$_{1-9}$-specific CD8$^+$ T cell clone from the PBMCs of HLA-A2$^+$ individuals with long-term T1D. INS-DRiP$_{1-9}$ specific T cells were detected by dual HLA-A2$^{INS\text{-}DRiP}{}_{1-9}$ tetramer staining after periodic peptide-specific stimulation (upper panel). The frequency of INS-DRiP1-9-specific T cells prior to sorting is indicated in the dot plot. Specificity of tetramer-sorted CD8$^+$ T cells was validated upon expansion (lower panel). (c) Chromium release assay performed on JY cells loaded with either INS-DRiP$_{1-9}$ (black squares) or a non-relevant peptide (SP-PPi$_{15-24}$) (open circles) in presence of INS-DRiP$_{1-9}$ CTLs. Each E:T ratio was measured in triplicate. (d) Cytokine secretion by INS-DRiP$_{1-9}$-directed CTLs after co-culture with INS-DRiP$_{1-9}$ loaded JY cells. Data are shown as the mean ± s.d. (n=2 biological replicates). (e) Beta cell specific killing assay performed on dispersed human islet cells maintained in normal medium (homeostatic conditions; 5 mM glucose) (left panel) or inflammatory conditions (20 mM glucose, 1,000 U/ml IFN-γ and 2 ng/ml IL-1β) (right panel). Results are shown as % of beta cell death (% of decreased luciferase) after 48 h coculture with PPI-directed CTL (open triangles), pp65CMV-directed CTL (open circles) or INS-DRiP$_{1-9}$-directed CTL (black squares). Data are shown as the mean ± s.d. (Representative results of n=4 from each pancreas donor, n=4). Statistical analysis was performed using a two-way ANOVA with Holm-Sidak's multiple comparison test relative to the pp65 CMV-directed CTLs. (f) Percentage of beta cell death observed in two different pancreas preparation after incubation with INS-DRIP$_{1-9}$-specific CTLs in homeostatic or inflammatory condition (E:I ratio 1:5). (g) Cytokine secretion (IFN-γ, TNF-α, and MIP-1β) by INS-DRiP$_{1-9}$-specific CTLs after coculture with HLA-A2$^+$ human islet cells in low-glucose culture conditions. Data are shown as the mean ± s.d. (representative results from n=3 from each pancreas donor).

CTLs specific for this epitope are able to kill human beta cells *in vitro*. The presence of phenotypically naïve DRiP$_{1-9}$-specific CTLs in healthy individuals and memory T cells in individuals with T1D indicates that neoantigen-specific CTLs are part of the normal T cell repertoire, and that the higher frequency and activated phenotype of these CTLs detected in PBMCs of individuals with T1D patients points to peripheral activation linking DRiP$_{1-9}$-specific CTLs with the immunopathogenesis of T1D.

Our study proposes a new pathway of beta cell destruction by the immune system in which the generation of the neoepitope, such as INS-DRiP$_{1-9}$, plays a central role. The mechanisms regulating the synthesis of the INS-DRiP polypeptide are still unknown, but two scenarios can be envisaged. In the first model, exon 2 of the insulin gene would be spliced from the pre-mRNA, thus positioning the AUG$_{341}$ as the first AUG encountered by the ribosome during scanning. Alternatively, the ribosome scan-through of the canonical AUG may result in translation initiation at a downstream AUG in the mature insulin mRNA. Since we could not detect such splicing variants in mRNA analysis of human pancreatic islets under either normal or pathogenic conditions (supplementary figure 4), we favour the alternative translation initiation as the most plausible mechanism. Although the results obtained on 293T cells should be interpreted with some caution, environmental modifications leading to ER stress appear to be an important component that can control expression of

alternative reading frame as described for other stress-induced proteins (i.e., ATF4 and ATF5) [39, 40]. The fact that human beta cells are exceptionally sensitive to stress ER reinforces this finding [41]. In addition to an effect on translation initiation processes, environmental stress may also have an impact on the degradation of insulin byproducts. The absence of an in-frame stop codon in the INS-DRiP potein is peculiar and may imply the participation of distinct elimination mechanisms that are engaged in clearing nonstop proteins[42, 43]. Yet, we cannot exclude the possibility that the increased beta cell death observed in pathophysiological condition results also from an increase in HLA-peptide complexes at the beta-cell surface, similar to those seen in insulitic lesions found in the pancreases of individuals with type 1 diabetes[30]. Nevertheless, the implication of translational mistakes in autoimmunity begs for revision of transcriptome-based approaches for epitope discovery and offers alternatives for tissue- and antigen-specific therapeutic approaches aiming at the induction of immune tolerance. Our findings further support the emerging concept that beta cells are destroyed in T1D by a mechanism comparable to classical antitumor responses whereby the immune system has been trained to survey dysfunctional cells in which errors have accumulated[1].

MATERIALS AND METHODS

Prediction of alternative translation and HLA binding.
Candidate alternative translation initiation sites were identified using NetStart 1.0 software[44]. Potential HLA-A2 binding peptides were predicted by prediction algorithms NetMHC 3.4 [45].

Proteins and Peptides.
Human recombinant proteins GAD65, IA-2 and PPI were synthesized as previously described[46]. Recombinant INS-DRiP R-P and INS-DRiP C-H were obtained from human islet cDNA by PCR, consisting of the 43 amino acids encoded within the insulin mRNA excluding the poly(A) tail. These sequences were subsequently cloned into a bacterial expression vector Gateway cloning technology (Invitrogen, Carlsbad, CA, USA) creating an N-terminal histidine tag, used for protein purification on nickel column (GE Healthcare, #17531801). Peptides were synthesized by solid-phase Fmoc chemistry and validated by

ultra-performance liquid chromatography and mass spectrometry. Peptides (purity > 85%) were dissolved in 5% DMSO/PBS to a 1mM stock solution.

Peptide binding assays.

HLA class II binding affinity of peptides was measured in a cell-free competitive peptide binding assay[47, 48]. Binding competition between an increasing amount peptide of interest (0-300 μM) and a fixed amount of biotinylated reporter peptide (0.6 μM) in HLA-DQ2 and HLA-DQ8 molecules isolated from transduced 293T cells was determined. After overnight incubation at 4°C, plates were washed, and europium-streptavidin in assay buffer was added to each well followed by incubation while shaking. After washing, wells were incubated with enhancement buffer. Plates were read using a time-resolved fluorometer (1234, Wallac). IC_{50} values were calculated using Graphpad software employing a Sigmoidal dose-response (variable slope) equation based upon the observed binding of the tested peptides against the fixed concentration indicator peptide. The concentration of test peptide required for half-maximal inhibition of binding of the reporter peptide indicates the IC_{50} value. IC_{50} < 1 μmol/L is considered strong binding, between 1-10 μmol/L, between 10-100 μmol/L and above 100 μmol/L are considered intermediate, weak and no binding, respectively.

Generation and pulsing of human DCs.

Monocytes were isolated from buffy coats of homozygous HLA-DQ2, homozygous HLA-DQ8 or heterozygous HLA-DQ2/8 donors using $CD14^+$ Macs sorting (Miltenyi Biotec, #130050201) and monocyte-derived DCs were generated as described previously[49]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and subsequently $CD14^+$ monocytes were isolated and cultivated with GM-CSF (800 U/ml) and IL-4 (500 U/ml) (invitrogen, Breda, the Netherlands) for 6 d to obtain immature DCs (iDCs). These iDCs (0.5 x 10^6/well) were pulsed with recombinant INS-DRiP polypeptide (10 μg/mL) in the presence of lipopolysaccharide (100 ng/ml) and IFN-γ (1000 U/ml) to induce DC maturation. After 30 h, the pulsed and matured DCs were harvested, washed with PBS and lysed in 1ml lysis buffer (50 mM Tris, 150mM NaCl, 5 mM EDTA, 0.5% Zwitterion, 10 mM iodoacetamide and a complete protease inhibitor mix (Roche Applied Science). To remove nuclei and insoluble material the lysates were centrifuged for 60 min at 10,000 x *g*.

Peptide elution of HLA class II and mass spectrometry analysis.

The HLA-DQ molecules were extracted from the DC lysates by affinity purification[50, 51]. Lysates were precleared with Sepharose beads and mixed with Sepharose beads coupled with a pan-DQ antibody (purified from SPV-L3 hybridoma). After 60 min of incubation the beads were washed with 5 bed volumes lysis buffer subsequently followed by 4 bed volumes of low salt buffer (120 mM NaCl, 20 mM Tris-HCl, pH 8.0), 8 bed volumes of high salt buffer (1 M NaCl, 20 mM Tris-HCl, pH 8.0), 4 bed volumes of no salt buffer (20 mM Tris-HCl, pH 8.0), and 4 bed volumes of low Tris buffer (10 mM Tris-HCl, pH 8.0). The HLA-peptide complexes were eluted with 5 bed volumes of 10% acetic acid. The eluate was applied on a small homemade C18 column and eluted in two fractions with 20 and 30% acetonitrile. Subsequently, the HLA peptides were analyzed via on-line C18-nanoHPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo Scientific), and a Q Exactive mass spectrometer (Thermo Scientific). Fractions were injected onto a homemade precolumn (100 µm x 15 mm; ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm x 50 µm; ReproSil-Pur C18-AQ, 3 µm). The gradient was run from 0 to 30% solvent B (10:90:0.1 water/acetonitrile/formic acid [v/v/v]) in 120 min. The nano-HPLC column was drawn to a tip of ~5 µm and acted as the electrospray needle of the MS source. The Q Exactive mass spectrometer was operated in top10 mode. Parameters were resolution 70,000 at an automatic gain control target value of 3 million maximum fill time of 100 ms (full scan), and resolution of 35,000 at an automatic gain control target value of 1 million/maximum fill time of 128 ms for MS/MS at an intensity threshold of 780,000. Apex trigger was set to 1–5 s, and allowed charges were 1–3. All fractions were measured twice. In a postanalysis process, raw data were converted to peak lists using Proteome Discoverer 1.4. For peptide identification, MS/MS spectra were submitted to the human IPI database supplemented with INS-DRiP amino acid sequence using Mascot Version 2.4 (Matrix Science) with the following settings: 10 ppm and 20 millimass units deviation for precursor and fragment masses, respectively; no enzyme was specified. All reported hits were assessed manually, and peptides with Mascot scores <35 were generally discarded.

Blood Donors and Genotyping.

After informed consent, blood was collected from 46 new-onset T1D patients (Supplementary Table 1). PBMCs isolated from donors with celiac disease (CD) were used as an age- and HLA-DQ matched control cohort (n=9). The genotype of the T1D blood donors was determined by PCR. PCR was performed in a total volume of 25 µl, using 50 ng of genomic DNA and 10 pmol of each primer. The amplification buffer consisted of 1x Flexi Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 0.5 unit of Taq DNA polymerase (all reagents were obtained from Promega). PCR was carried out in a Peltier Thermal Cycler (PTC-200; MJ Research). After initial denaturation at 95 °C for 5 min, 35 cycles were run, consisting of denaturation at 94 °C for 30 s, annealing at 60 °C (rs3842752) or 65 °C (rs3842753) for 30 s and extension at 72 °C for 30 s. Finally, a 5 min extension step was performed at 72 °C. For visualisation, 5 µl of the amplification products were run on a 2% agarose MP gel (Boehringer Mannheim) prestained with ethidium bromide.

Genotyping was assessed by PCR using a common forward primer (5'-TGGGGCAGGTGGAGCT-3') and allele-specific reverse primers. The presence of allele A or G in SNP rs3842752 was determined using 5'-(T/G) GGGGCTGCCTGCA-3' or 5'-(T/G)GGGGCTGCCTGCG-3', respectively. The presence of allele T or G in SNP rs3842753 has been determined using 5'-GAGGCGGCGGGTGT-3' or 5'-GAGGCGGCGGGTGG-3' reverse primers, respectively.

T cell proliferation assay.

Freshly isolated PBMCs were used to investigate the immunogenicity of recombinant INS-DRiP polypeptide in a T cell proliferation assay[52]. PBMCs were seeded (150,000/well) in flat-bottomed 96-well microculture plates (Greiner, Nürtingen, Germany) and cultured for 5 days at 37°C in 5% CO2, in a humidified atmosphere. Cells were cultured in triplicates in medium alone, with 10 µg/mL recombinant INS-DRiP polypeptide, or recombinant IL-2 10% (25 units/mL; Genzyme, Cambridge, MA) as positive control. In the final 16 h of culture, 50 µL RPMI 1640 (Dutch modification; Gibco) containing 0.5 µCi ^{3}H-thymidine (DuPont NEN, Boston, MA) was added per well. After the cells were harvested on filters with an automated harvester, proliferation was determined by the measurement of ^{3}H-thymidine incorporation in an automatic liquid scintillation counter. All results are calculated as mean counts per minute (CPM) in the presence of antigen and compared with medium alone.

Detection of epitope-specific CD8+ T cells in PBMCs of T1D patients.
Heparinized blood samples were drawn from 24 T1D patients (including new onset patients) and 13 sex- and age-matched healthy controls (Supplementary table 2).PBMCs were isolated by Ficoll-isopaque density gradient centrifugation and frozen in liquid nitrogen until use. For the detection of epitope-specific $CD8^+$ T cells, thawed PBMCs were stained with Qdot-labeled multimeric complexes. Peptide-HLA-A2 (pHLA-A2) monomers and multimeric pHLA-A2 complexes were generated as previously described [27]. Samples with $CD8^+$ T cell counts under 50,000 were excluded from analysis. Experiments were performed blinded. The study protocol was approved by the Leiden University Medical Center institutional review board.

Cell culture.
293T cells were obtained from the institutional cell line collection. Their identity was confirmed by the Forensic Laboratory for DNA Research, Department of Human Genetics, Leiden University Medical Centre, by short tandem repeat analyses and comparison with the STR databases. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% heat inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. JY cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 8% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Validation for HLA-A2 surface expression was done by FACS prior to every experiments (BB7.2, BD Pharmingen, #551285). All cells have been tested negative for mycoplasma by mycoplasma detection kit Lonza (Walkerville, MD). DCs were maintained in RPMI-1640 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, glutamax and 10% FCS. For the maturation of DCs during pulsing, additional cytokines were added to the medium (see above). For maintenance and expansion autoreactive $CD8^+$ T cell clones were specifically stimulated every 14 d with irradiated allogeneic PBMCs and irradiated peptide-pulsed HLA-A2-expressing JY cells in IMDM supplemented with a mixture of cytokines similarly as described below.

Human pancreatic islet isolation.
Human islets were isolated from brain-dead organ donors. Purified islets used in these experiments were unsuitable for clinical islet transplantation as described previously[38]. Human islet isolations were performed in the

institutional GMP-facility. Purity of the final islet preparation was assessed by 1 mmol/l dithizone (Sigma-Aldrich) staining and ranged from 45-95%. Purified islets were cultured in CMRL-1066 medium supplemented with 10% human serum, 20 µg/ml ciprofloxacin, 50 µg/ml gentamycine, 2 nmol/l L-glutamine, 250 ng/ml fungizone, 10 mmol/l HEPES and 10 mmol/l nicotinamide.

Generation and characterization of DRiP$_{1-9}$-specific CD8$^+$ T cell clone.
Freshly isolated PBMCs from a long-term HLA-A2$^+$ individual with T1D (clone#1) and from an individual with new-onset T1D (clone#2) were seeded 150,000 cells/well with 10 ug/ml DRiP$_{1-9}$ peptide in IMDM supplemented with 10% human serum, 0.5% LeucoA, 0.1 ng/ml IL-12, 10 ng/ml IL-7, 25 U/ml IL-2 and 5 ng/ml IL15. After 14 d of culture, 20,000 cells/well were restimulated specifically with 20,000 cells/well irradiated DRiP$_{1-9}$ peptide-pulsed HLA-A2-expressing JY cells (2 µg/ml peptide with 10x10^6 cells in AIM-V medium (Life Technologies) for 2 h at 37˚C and 100,000 cells/well irradiated allogeneic PBMCs in IMDM medium supplemented with human serum and cytokines as described above. On day 28, CD8$^+$ T cells were double stained with DRiP$_{1-9}$ tetramers (Tm), single cell sorted into round bottom 96-well plates and restimulated as described. Expanding CD8$^+$ Tm$^+$ T cell clones were isolated and restimulated every 14 d. Every 4-5 d fresh IMDM supplemented with 25 U/ml IL-2 and 10 ng/ml IL-15 was added to the culture media.

Lentiviruses production and transduction.
Third-generation self-inactivating lentiviral vectors were produced as described previously [53]. HIV p24 concentration was measured with antigen capture ELISA (ZeptoMetrix, Buffalo, NY); 1 ng p24 corresponds to 2500 infectious particles. Human pancreatic islets were dissociated and dispersed prior transduction. The islets were incubated with 0.05% trypsin for 5-10 minutes at 37°C and subsequently passed through a 40 µm filter. Immediately after dispersion the islets were transduced with HIP-Luc2CP containing lentivirus at MOI=2. The medium was refreshed after overnight incubation. Cells were maintained in culture for 2 d prior incubation with CTL. Human pancreatic islets were maintained in DMEM low glucose supplemented with 8% heat inactivated FBS, 100 u/ml penicillin and 100 µg/ml streptomycin in ultra-low attachment plates as described previously[38].

Polyethylenimine transfection.
293T cells were transfected in suspension using polyethylenimine (PEI). 3 µg of PEI (pH 7.4) was used per µg DNA used in the transfection mix supplemented with Opti-MEM I reduced serum medium and incubated for 10 minutes at room temperature (RT) before adding to the cell suspension. After overnight incubation the medium was refreshed and further experiments were performed.

DNA constructs.
pLV-CMV-PPI-GFP and pLV-CMV-PPI-DRiP-GFP have been generated from the intermediate cloning vectors pJET1.2-PPI and pJET1.2-DRiP, respectively. pJET1.2-PPI has been generated by cloning PPI (5'UTR to PPI termination codon) into pJET2.1/blunt vector using the primers: Fw 5'-AGCCCTCCAGGACAGGC-3' and Rv 5'GTTGCAGTAGTTCTCCAGCT-3' on human cDNA. pJET1.2-DRiP was generated by cloning PPI (5'UTR to 3'UTR) into pJET2.1/blunt vector using the primers: Fw 5'-AGCCCTCCAGGACAGGC-3' and Rv 5'- TTTTGCTGGTTCAAGGGCTTTATT-3' on human cDNA. The Insulin and INS-DRiP fragment were subcloned into pEGFP-N1 (Clontech) in frame with the GFP ORF to generate PPI-GFP and INS-DRiP-GFP expressing vectors and subcloned into pLV-CMV vectors. pLV-DRiP-GFP was generated by cloning the XhoI/HpaI DRiP-GFP fragment from the pLV-CMV-INS-DRiP-GFP construct using DRiP Fw 5'- CGCTCGAGTAAATGCTGTACCAGCATCTGC-3' and GFP Rv 5'-CGGTTAACAACTTACTTGTACAGCTCGTCC-3' primers into pLV-CMV vector.

Cytotoxicity assays.
Chromium release assay were performed as previously described[38]. Briefly, JY cells loaded with INS-DRiP$_{1-9}$ or SP-PPI$_{15-24}$ peptide were incubated with 100 µl Na-chromate (51Cr, 3.7 MBq) and seeded in triplicate for 1 h, at various effector-to-target (E:T) together with CTLs. After 16 h incubation at 37°C in 5% CO2, supernatants were collected, and the release of 51Cr was measured with a gamma-counter (Wallac/PerkinElmer, Waltham, MA, USA). Spontaneous and maximum releases were obtained by incubation with medium and 1% triton in PBS, respectively. The specific lysis was calculated as percentage of specific lysis = 100 × (experimental release – spontaneous release) /(maximum release – spontaneous release).

To analyze specific beta cell death, human islet cells were transduced with LV- HIP-Luc2CP lentivirus vector as described previously[38]. Transduced islet

cells were incubated with increasing amounts with epitope-specific $CD8^+$ T cells in IMDM supplemented with 5% HS, 25 U/ml IL-2 and 10 ng/ml IL-15 for 48 h. Experiments were performed with different effector-to-islet cells target ratio (E:I ratio) and performed in fourfold. Subsequently, cells were lysed in luciferase lysis buffer (25 mM Tris/HCl pH 7,8, 2mM CDTA, 2mM DTT, 10% glycerol, 1% Triton X-100) and T cell mediated target cell killing was verified by measuring light emission using Luciferase Assay Reagent (Promega) and Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). Results are shown as decrease luciferase activity. The percentage of beta cell death is calculated using the following formula: $100-[(RLU_{CTLx}/ RLU_{average\ CMV\ CTL})x100]$.

Protein analysis.

Protein lysates were analyzed on SDS-PAGE. Before loading, samples were boiled in sample buffer (2% SDS, 25mM Tris-HCl pH 6,8, 1,5 mM Bromophenol blue, 0.14 mM β-mercaptoethanol, 10% glycerol) for 5 minutes at 96˚C. Proteins were transferred to Immobilon-P (Immobilon-P^{SQ} transfer membrane, pore size 0.2 µm (polyvinylidene difluoride); Millipore, Etten-Leur, The Netherlands) and visualized by standard antibody staining protocols for anti-Green Fluorescent Protein (1:2000, Life technology, #A11122), anti-insulin (1:1000 clone H-86, SantaCruz sc-9168)and anti-actin (1:5000 clone C4,MP Biomedicals, #691001). GFP fusion proteins and insulin were analysed on 10% gels and 15% gels, respectively. For INS-DRiP-GFP detection, 100 µg of protein from cells transfected with INS-DRiP-GFP was loaded on 10% gel for GFP detection owing to low DRiP expression, while 30 µg were loaded on the corresponding 15% gels for insulin detection.

Immunohistochemistry.

Forty-eight hours post transfection, 293T cells were fixed in 4% paraformaldehyde supplemented with 0,1% saponin. Blocking was done with PBS/BSA 1%, and first and secondary antibodies were diluted in PBS/BSA 1%. Anti-proinsulin (Monoclonal Human Pro-Insulin, Beta cell biology consortium) was used at 1:500 and secondary antibody coupled to Alexa 568 was used at a dilution of 1:500. Nuclei were stained with Hoechst. Cells were analysed by confocal microscope (Zeiss).

Statistical analyses.

Data are presented as mean ± s.d. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical tests used for each experiment are indicated in the legend of the corresponding figures. P values <0.05 were considered statistically significant. The number and the type of replicates are indicated in the legend of the figures.

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Contributions

B.O.R., R.C.H. and A.Z. conceived and directed the project and wrote the manuscript. M.J.L.K. performed the key experiments and analyzed the data. P.A.v.V. performed mass spectrometry experiments. A.M.J. and S.L. cloned $DRiP_{1\text{-}9}$-specific CTLs. M.v.L., T.N. and A.R.v.d.S. selected patient samples, performed ELISPOT assay and characterized $CD8^+$ clones. F.C. and E.J.P.d.K. provided human islets.

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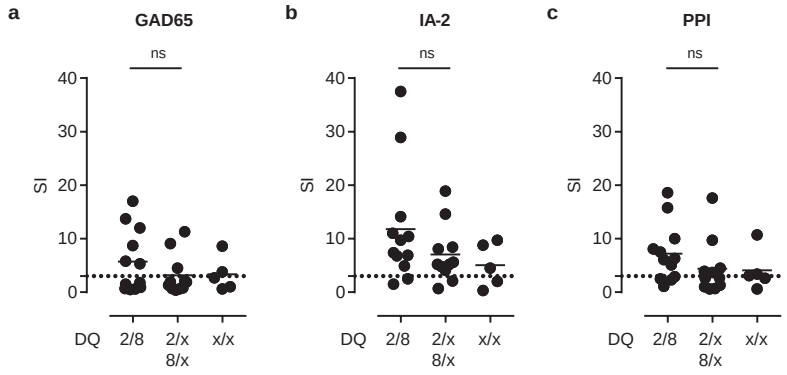
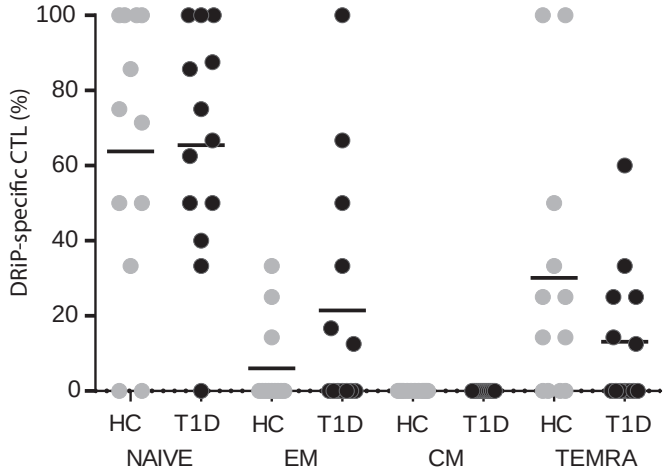
SUPPLEMENTARY INFORMATION



Supplementary figure 1: Lymphocyte proliferation responses to native beta cell proteins. PBMCs isolated from fresh whole blood of T1D patients with the high risk haplotype DQ2/8, DQ2/x or DQ8/x, and DQx/x were incubated with a) recombinant GAD65 b) recombinant IA-2 or c) recombinant PPI. The x represents any HLA-DQ haplotype except DQ2 or DQ8. P-values have been calculated by a Mann-Whitney t test.

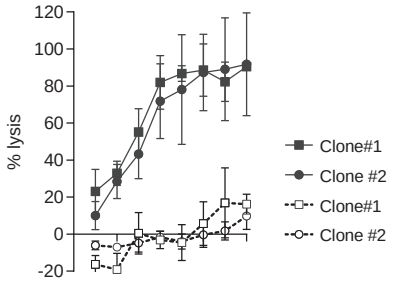


Supplementary figure 2: Phenotypical analysis of DRiP Tm+ CD8 T cells using CCR7 and CD45 surface markers. The T cell phenotypes are defines as naïve (CCR7+/CD45RA+), central memory (CM) (CCR7+/CD45RA-), effector memory (EM) (CCR7-/CDRA-), or terminally differentiated effector memory (TEMRA) (CCR7-/CD45RA+). The graph represent the percentage of each phenotype within the DRiP Tm^+ CD8 T cell population of T1D patients (black circles) and matched healthy control subjects (grey circles).

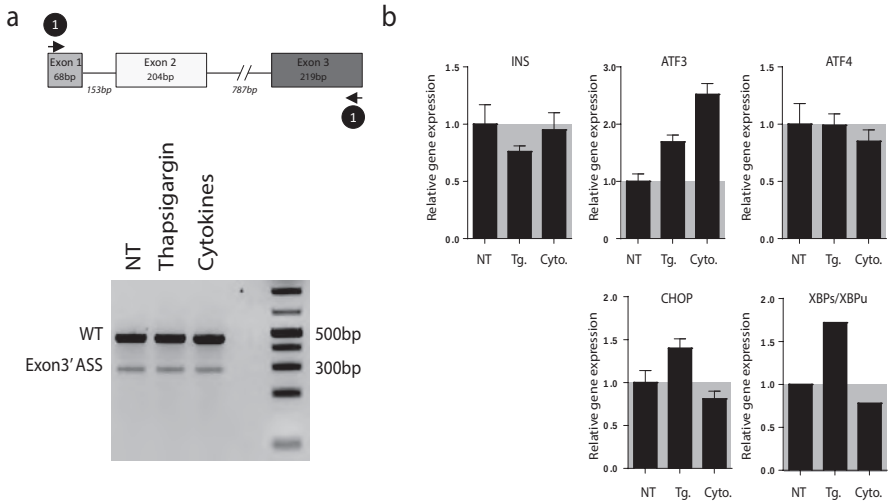
a

	TRAV	TRAJ	CDR3 Junction	TRBV	TRBD	TRBJ	CD3 Junction
Clone#1	12-2*03	34*01	CAVNKTDKLIF	6-1*01	1*01	1-2*01	CASSVTGNGYTF
Clone#2	10*01	8*01	CVVNMNTGFQKLVF	12-3*01/12-4*01	1*01	2-2*01	CASSPPQGGNTGELFF

b



Supplementary figure 3: T cell receptor analysis of DRiP$_{1-9}$-specific T cell clones. a) TCRα chain and TCRβ chain sequences of the two DRiP$_{1-9}$-specific clones as analysed by IMGT/V-QUEST. b) Chromium release assay performed on JY cells loaded with DRiP$_{1-9}$ peptide (solid lines and black symbols) or CMV peptide (dashed lines and open symbols) using the two DRiP$_{1-9}$-specific clones (circles and squares). Data represent mean ± standard deviation (n=3).



Supplementary figure 4: mRNA analysis of human islets. A) PCR analysis performed on RNA extracted from human islets maintained in ER stress condition (24h-0.1μM Thapsigargin) or inflammatory conditions (24h-IFN$_γ$/IL1β) using primer set 1 covering the whole insulin mRNA sequence as indicated by the arrows on the INS gene schematic representation (upper panel). The upper band shows expression of the correctly spliced mRNA (WT), the lower represents an alternative transcript resulting from the use of an alternative splicing site (ASS) within exon 3. (b) Insulin and ER stress genes expression in human islets following thapsigargin treatment and cytokine mix stimulation assessed by qPCR. GAPDH was used as housekeeping gene.

Supplementary table 1: Haplotyping, LST and Elispot data T1D cohort and CD controls. Patient genotypes were determined by PCR. LST was performed with freshly isolated PBMCs with recombinant polypeptides of native beta cell proteins GAD65, IA-2 and PPI, recombinant INS-DRiP C-H polypeptide (C-H) and recombinant INS-DRiP R-P polypeptide (R-P). GrzB and IFNγ secretion upon INS-DRiP$_{1\text{-}9}$ (peptide) stimulation in PBMCs were determined using ELISPOT. Data were corrected by the background signal (bkgd) which was determined in absence of peptide. Since ethical restrictions precluded testing healthy children, we referred to children suspected of having celiac disease for comparison, as these represent similar age, HLA and chronic inflammation as control cohort. Patients indicated in blue have either a protective DQ6.2 or INS-SNP C-H phenotype. SI ≥ 3 are considered positive (bold values); ND: not determined as a result of insufficient donor PBMCs.

Patients	Haplotype		Single Nucleotide Polymorphism						Lymphocyte Stimulation Test					Elispot					
															Granzyme B			Interferon gamma	
	HLA A	HLA DQ	genotype				INS-DRiP							bkgd	peptide	peptide	bkgd	peptide	peptide
			rs3842752		rs3842753				GAD65	IA-2	PPI	DRiP-CH	DRiP-RP	(#)	(#)	(SI)	(#)	(#)	(SI)
1	3/24	6.3/9	G	G	G	G	R-P	R-P	2,7	2,0	2,6	**5,1**	ND	ND	ND	ND	ND	ND	ND
2	2/30	2/8	G	G	G	G	R-P	R-P	0,6	**4,9**	2,3	1,4	ND	ND	ND	ND	ND	ND	ND
3	24/36	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	7	**7**	1	1	1
4	3/3	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	3	**3**	1	10	**10**
5	1/2	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	0	0	1	0	0
6	3/24	2.2/8	G	G	T	G	R-H	R-P	ND	ND	ND	ND	ND	1	5	**5**	6	9	2
7	1/29	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	0	0	1	1	1
8	1/2	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	1	1	1	2	2
9	11/31	5/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	1	1	1	0	0
10	1/29	2/2	G	G	G	G	R-P	R-P	**4,5**	**5,2**	2,7	**9,8**	**3,7**	1	5	**5**	1	1	1
11	2/31	5/8	G	G	G	G	R-P	R-P	0,7	2,1	1	**3,5**	1,4	1	5	**5**	1	0	0
12	2/2	5/8	G	G	T	G	R-H	R-P	ND	ND	ND	ND	ND	4	11	**3**	8	12	2
13	2/24	2/8	G	G	G	G	R-P	R-P	0,9	**7,4**	**6,1**	**8,1**	**4,5**	1	0	0	1	3	**3**
14	24/33	2/9	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	1	1	1	1	1
15	2/2	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	0	0	25	28	1
16	2/24	2/8	G	G	T	G	R-H	R-P	ND	ND	ND	ND	ND	1	3	**3**	1	0	0
17	24/32	4/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	3	**3**	1	0	0
18	1/30	2/2.2	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	11	11	1	1	1	1
19	1/2	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	2	2	1	1	1	1

Supplementary table 1: Haplotyping, LST and Elispot data T1D cohort and CD controls. continued

Patients	Haplotype		Single Nucleotide Polymorphism						Lymphocyte Stimulation Test					Elispot					
															Granzyme B			Interferon gamma	
	HLA A	HLA DQ	genotype				INS-DRiP							bkgd	peptide	peptide	bkgd	peptide	peptide
			rs3842752		rs3842753				GAD65	IA-2	PPI	DRiP-CH	DRiP-RP	(#)	(#)	(SI)	(#)	(#)	(SI)
20	2/23	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	9	4	0	3	2	1
21	30/33	2/8	G	G	G	G	R-P	R-P	**5,3**	**6,8**	**6,3**	**12,4**	**12,2**	1	18	**18**	1	0	0
22	24/30	2/8	G	G	T	G	R-H	R-P	**8,7**	**10,4**	**7,5**	**16,3**	**13,8**	2	1	1	1	4	**4**
23	1/1	2/2	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	1	0	0	2	0	0
24	3/29	6/8	G	G	T	G	R-H	R-P	0,6	**4,1**	0,7	1,6	0,6	1	0	0	1	2	2
25	1/29	2/5	G	G	G	G	R-P	R-P	1,9	**5,2**	1,3	0,4	0,4	1	3	**3**	1	0	0
26	32/68	5/8	G	G	G	G	R-P	R-P	**9,1**	**14,6**	**9,7**	**13,4**	**9,2**	ND	ND	ND	ND	ND	ND
27	2/68	8/8	G	G	G	G	R-P	R-P	D	ND	ND	ND	ND	1	1	1	6	0	0
28	3/3	5/8	G	G	G	G	R-P	R-P	1,3	**4,8**	2,6	**10,7**	**6,4**	1	1	1	1	0	0
29	1/2	6/7	A	G	T	G	C-H	R-P	**3,8**	**4,5**	**3,4**	**5,8**	1,2	ND	ND	ND	ND	ND	ND
30	1/2	2/8	G	G	T	G	R-H	R-P	**5,8**	**28,9**	**8,1**	**35,1**	**18,8**	ND	ND	ND	ND	ND	ND
31	11/30	2/7	G	G	G	G	R-P	R-P	2,1	**8,1**	**4,5**	1,0	0,6	ND	ND	ND	ND	ND	ND
32	24/34	5/5	G	G	G	G	R-P	R-P	1,0	**8,8**	**3,1**	1,6	**4,8**	ND	ND	ND	ND	ND	ND
33	2/2	2/8	A	G	T	G	C-H	R-P	0,5	2,5	1,1	0,7	0,5	ND	ND	ND	ND	ND	ND
34	3/24	2/8	G	G	G	G	R-P	R-P	1,5	**9,7**	2,9	**12,6**	**4,3**	1	4	**4**	1	0	0
35	2/31	2/4	A	G	T	G	C-H	R-P	0,8	**8,4**	3,7	**4,2**	0,6	1	0	0	1	0	0
36	1/1	2/8	G	G	T	G	R-H	R-P	**12,0**	**11,0**	**10**	**18,6**	**17,9**	ND	ND	ND	ND	ND	ND
37	1/31	2/8	G	G	G	G	R-P	R-P	**17,0**	**14,1**	**15,8**	**22,6**	**38,0**	ND	ND	ND	ND	ND	ND
38	3/3	2/8	G	G	G	G	R-P	R-P	0,7	1,5	2,5	2,0	1,5	2	1	1	1	1	1
39	2/2	5/5	G	G	G	G	R-P	R-P	0,6	0,3	0,6	0,9	1,0	1	1	1	1	1	1
40	1/3	2/5	G	G	G	G	R-P	R-P	1,9	**5,6**	**3,9**	2,3	2,1	1	0	0	1	0	0
41	3/3	2/8	G	G	G	G	R-P	R-P	1,8	**6,9**	**5,1**	**10,0**	**8,9**	1	9	**9**	1	1	1
42	1/30	4/7	G	G	G	G	R-P	R-P	**8,6**	**9,7**	**10,7**	**4,3**	1,9	1	0	0	1	0	0

Supplementary table 1: Haplotyping, LST and Elispot data T1D cohort and CD controls. continued

Patients	Haplotype		Single Nucleotide Polymorphism						Lymphocyte Stimulation Test					Elispot					
															Granzyme B			Interferon gamma	
	HLA A	HLA DQ	genotype				INS-DRiP							bkgd	peptide	peptide	bkgd	peptide	peptide
			rs3842752		rs3842753				GAD65	IA-2	PPI	DRiP-CH	DRiP-RP	(#)	(#)	(SI)	(#)	(#)	(SI)
43	3/30	2/7	G	G	G	G	R-P	R-P	**11,3**	**18,9**	**17,6**	**11**	ND	1	0	0	1	0	0
44	24/32	2/8	G	G	G	G	R-P	R-P	ND	ND	ND	ND	ND	0	0	0	1	0	0
45	2/24	2/8	G	G	G	G	R-P	R-P	**13,7**	**37,5**	**18,6**	**54,5**	**34,8**	ND	ND	ND	ND	ND	ND
46	1/2	6.4/8	G	G	G	G	R-P	R-P	0,4	0,7	0,6	0,4	0,5	46	44	0	4	12	3
Controls	**Haplotype**		**Single Nucleotide Polymorphism**						**Lymphocyte Stimulation Test**					**Elispot**					
1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2	2	1	0	1	1
2	2/3	5/8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	0	0	0	0	0
3	1/1	2/2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	1	1	1	0	0
4	ND	2.2/7	ND	ND	ND	ND	ND	ND	**3.3**	**3.2**	0.8	1.2	0.8	2	0	0	0	0	0
5	1/24	2/5	ND	ND	ND	ND	ND	ND	**10.8**	**12.8**	**11.8**	**11**	**5.5**	0	0	0	7	0	0
6	3/3	2/6.2	ND	ND	ND	ND	ND	ND	**4.3**	**9**	**5.4**	2.2	1.4	0	0	0	12	28	2,3
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	1	1	0	1	1
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	1	1	0	1	1
9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	0	0	0	3	**3**

Supplementary table 2: *In silico* prediction of HLA-A2 binding peptides within INS-DRIP. a) Prediction of HLA-A2 binding peptides using NetMHC 3.4. Scores are predicted IC_{50} values in nM, binding threshold for strong binder (SB) < 50 nM (*) and weak binder (WB) 50 ≥ nM < 500 (**). † Position of peptide within the amino acid sequence of INS-DRIP. ‡Binding affinity is predicted using prediction algorithm NetMHC 3.4. Grey sequences represent peptides that are generated in case translation continues into the poly-A tail. b) Overview of the experimentally determined IC_{50} value, as determined by competition-based peptide binding assay of HLA-A2 peptide HBV cAG_{18-27}, $INS\text{-}DRiP_{1-9}$ and non HLA-A2 binding peptide MAGE A wt. † Binding affinity thresholds: strong binder < 5μM, intermediate binder 5≥μM<15, weak binder 15≥μM<100 and no binder >100μM. The IC50 value is calculated based on the competition between the test peptides and the high HLA-A2 affinity fluorescent indicator peptide.

a

Position†	Sequence	Predicted IC_{50} ‡ (nM)
1-9	MLYQHLLPL	6*
2-10	LYQHLLPLP	24974
3-11	YQHLLPLPA	475**
4-12	QHLLPLPAG	25386
5-13	HLLPLPAGE	16794
6-14	LLPLPAGEL	5110
7-15	LPLPAGELL	22833
8-16	PLPAGELLQ	28830
9-17	LPAGELLQL	22086
10-18	PAGELLQLD	31411
11-19	AGELLQLDA	27083
12-20	GELLQLDAA	19597
13-21	ELLQLDAAR	28213
14-22	LLQLDAARR	26337
15-23	LQLDAARRQ	23953
16-24	QLDAARRQP	29197
17-25	LDAARRQPH	30297
18-26	DAARRQPHT	28620
19-27	AARRQPHTR	30415
20-28	ARRQPHTRR	31527
21-29	RRQPHTRRL	26408
22-30	RQPHTRRLL	18406
23-31	QPHTRRLLH	28981
24-32	PHTRRLLHR	30385
25-33	HTRRLLHRE	30716
26-34	TRRLLHRER	30360
27-35	RRLLHRERW	27774
28-36	RLLHRERWN	26736
29-37	LLHRERWNK	26569
30-38	LHRERWNKA	29808
31-39	HRERWNKAL	27819
32-40	RERWNKALE	29771
33-41	ERWNKALEP	28119
34-42	RWNKALEPA	16649
35-43	WNKALEPAK	29052

a continued

Position[†]	Sequence	Predicted IC_{50}[‡] (nM)
36-44	NKALEPAKK	30268
37-45	KALEPAKKK	28739
38-46	ALEPAKKKK	31642
39-47	LEPAKKKKK	31718
40-48	EPAKKKKKK	32481
41-49	PAKKKKKKK	33037
42-50	AKKKKKKKK	32158
43-51	KKKKKKKKK	31967

b

Peptide	Sequence	Experimental IC_{50} (nM)
HBV cAG_{18-27}	FLPSDFFPSV	1.1
MAGE A wt	YLEYRQVPG	128.0
$DRiP_{1-9}$	MLYQHLLPL	2.3

Supplementary table 3: Description of donors examined by Qdot analysis. Sex, age, disease duration and $CD8^+$ T cell counts and % of INS-$DRiP_{1-9}^+$ or $virus^+$ CD8 T cells of T1D patients, New onset (NO) T1D patients and healthy control subjects matched for HLA-A2, age and sex. Disease duration reflects the time between the diagnosis of T1D and the moment blood samples were taken for analysis. CD8 T cells were identified by flow cytometry. $CD8^+$ T cell counts below 50,000 were excluded from the graphs and analyses due to expected inaccuracy of T cell frequencies (frequencies (indicated by italic font and marked with *). NA, not applicable. NO, new onset, patients diagnosed within 90 days prior blood analysis. $NO^†$, new onset T1D patient tested within a month from diagnosis.

Sex	Age	Disease duration (months)	$CD8^+$ T-cells (#)	INS-$DRIP^+$ CD8 T-cells (%)	$Virus^+$ CD8 T-cells (%)
T1D patients					
female	*10*	*5*	*45,299*$^+$	*0.0044*	*0.0111*
male	8	12	84,762	0.0012	0.0237
male	17	15	76,037	0.0040	0.0595
female	10	30	207,338	0.0015	0.0053
male	11	6	179,447	0.0045	0.0039
male	23	6	79,898	0.0025	0.0190
female	15	13	151,787	0.0046	0.0040
female	13	18	71,630	0.0042	0.0070
male	10	23	147,814	0.0055	0.0089
male	10	5	215,161	0.0023	0.0136
male	10	13	182,324	0.0022	0.0011
male	14	18	210,107	0.0024	0.0010
male	13	24	150,974	0.0013	0.0013
male	18	19	214,158	0.0038	0.1101
male	*19*	*12*	*46,175**	*0.004*	*0.004*
male	24	6	84,387	0	0.007
male	20	6	83,466	0.004	0.089
New-onset T1D patients					
female	*14*	*NO*	*49,238*$^+$	*0.01*	*0.01*
female	35	NO	55,632	0	0.02
male	*21*	*NO*	*40,578**	*0.002*	*0.022*
male	13	$NO^†$	115,210	0.002	0.979
male	25	NO	76,212	0.005	0.004
female	13	NO	66,460	0.006	0.19
female	*19*	*NO*	*42,771**	*0.01*	*0.014*

Supplementary table 3: Description of donors examined by Qdot analysis. continued

Sex	Age	Disease duration (months)	$CD8^+$ T-cells (#)	INS-$DRIP^+$ CD8 T-cells (%)	$Virus^+$ CD8 T-cells (%)
Healthy controls					
female	10	NA	122,400	0.0016	0.0066
male	9	NA	207,458	0.0034	0.0271
male	17	NA	159,001	0.0025	0.0769
female	10	NA	109,319	0.0009	0
male	11	NA	211,298	0.0033	0.2037
male	23	NA	210,190	0.0024	0.0043
female	13	NA	164,197	0.0025	0.0472
female	*12*	*NA*	*49,202*[*]	*0.0062*	*0.0983*
male	11	NA	191,667	0.0005	0.0357
male	10	NA	117,310	0.0026	0.0043
male	10	NA	203,629	0.0005	0.001
male	14	NA	214,768	0.0019	0.0019
male	13	NA	134,032	0.0008	0.0135

SUPPLEMENTARY MATERIALS

Cytokine measurements

Detection of INS-$DRiP_{1-9}$–specific T cells secreting Granzyme B (GrzB) (U-Cytech, #CT229PR5) or interferon gamma (IFNγ) (U-Cytech, #CT230PR5) was determined by enzyme-linked immunospot (ELISPOT)[10, 54]. Freshly isolated PBMCs were incubated in the presence of 10 μg/mL peptide or diluent alone for 48 hrs. The cells were then transferred onto microtiter pre-coated with monoclonal anti–IFNγ or anti–GrzB capture antibody. Detection of cells producing IFNγ or GrzB was performed using biotinylated anti–IFNγ or anti–GrzB detector antibody. Data are expressed as the mean number of spots per triplicate divided by the mean number of spots in triplicate in the presence of diluent alone. Stimulation index values of ≥3 are considered as positive.

HLA-A2 peptide binding affinity

Binding affinity to HLA-A2 was determined by a competition-based cellular peptide binding assay. JY cells (HLA-A020) were stripped of their naturally bound peptides in ice-cold citric acid elution buffer (1:1 mixture of 0.263M citric acid and 0.12M Na_2HPO_4, PH 3.1). After 90 seconds incubation, the reaction was stopped by addition of ice-cold Iscove's modified Dulbecco's media (IMDM-2)

supplemented with 2% foetal calf serum (FCS). The cell pellet was resuspended in IMDM-2 supplemented with 2% FCS and 2µg/ml β2M at a concentration of $4x10^5$ cells/ml. An 8-fold dilution series of test peptides (final concentration of 100 µM to 0.8 µM was incubated with fluorescent-labelled reference peptide (150 nM) and $4x10^4$ cells for 24 hours at 4°C in the dark. Peptide binding affinity was determined by decrease in fluorescence intensity measured by FACS. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression analysis using the following formula: inhibition (%) = $((MF-MF_{min})/(MF_{max}-MF_{min}))*100$.

Reverse-transcription PCR and real-time PCR.

Total cellular RNA was extracted from human islets stimulated with cytokine mix (IFNγ: 1000U/ml; IL1β: 2ng/ml) or thapsigargin (0.1uM) using Trizol. 200ng RNA was reverse transcribed using Superscript RT II kit (Invitrogen, Karlsruhe, Germany). Expression pattern of Insulin was addressed by regular PCR using INS Fw5'-AGCCCTCCAGGACAGGC-3'andINSRv5'-TTTT'GCTGGTTCAAGGGCTTTATT-3'. Expression of the genes of interest was detected using the following primers:
Insulin
Fw 5'-GCAGCCTTTGTGAACCAACA-3', Insulin Rv 5'-CGGGTCTTGGGTGTGTAGAAG-3';
ATF3
Fw 5'- GTGCCGAAACAAGAAGAAGG-3', ATF3 Rv 5'- TCTGAGCCTTCAGTTCAGCA-3';
ATF4
Fw 5'- GGTCAGTCCCTCCAACAACA-3', ATF4 Rv 5'- AGGTCATCTGGCATGGTTTC-3';
CHOP
Fw 5'- GACCTGCAAGAGGTCCTGTC-3', CHOP Rv 5'- CTCCTCCTCAGTCAGCCAAG-3';
XBP
Fw 5'- GGAGTTAAGACAGCGCTTGG-3', XBP Rv 5'- CTGCAGAGGTGCACGTAGTC-3'; XBPs
Fw 5'- CTGAGTCCGCAGCAGGTG-3', XBPs Rv 5'- GAGATGTTCTGGAGGGGTGA-3';
GAPDH
Fw 5'-ACAGTCAGCCGCATCTTCTT-3', GAPDH Rv
5'-AATGAAGGGGTCATTGATGG-3'.

Real-time PCR was performed in triplicate using the SybrGreen master mix kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems Step One Plus machine. GAPDH mRNA was used as reference.

TCR sequencing

Total RNA was isolated using Nucleospin miRNA (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer's protocol. Oligo(dT)15-primed (Promega, Madison, WI) first-strand complementary DNA was synthesized from 100ng total RNA template using superscript III Reverse Transcriptase (Life Technologies Ltd, Paisley, UK) in 20 µl according to manufacturer's protocol.
Polymerase chain reaction was performed to determine the TCR VA and VB distribution using forward primers that cover the complete TRAV and TRVB repertoire, together with reverse primers specific for the TRAC (5'-AGCAGATTAAACCCGGCCA-3' and TRBC 5'-TTCTCTTGACCATGGCCATC-3' constant region. PCR amplification was performed in a volume of 10 µl, containing 0.5 ng cDNA, 1x Green Gotaq Flexi Buffer (Promega, Madison, WI), 5 pmol of each primer, 200 µM dNTPs (Promega, Madison, WI), 1.5 mM MgCl2 (Promega, Madison, WI) and 0.5 unit of GoTaq G2 Flexi DNA Polymerase (Promega, Madison, WI). An initial denaturation step at 95 °C for 2 minutes, was followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 65 °C (TRAV) or 60 °C (TRBV) for 30 seconds and extension at 72 °C for 30 seconds. Finally, a 5 minute extension step was performed at 72 °C. The amplification products were run on a 1.5% (w/v) agarose MP gel (Roche Diagnostics GmbH, Mannheim, Germany) pre-stained with ethidium-bromide.

TRAV12 and TRBV6 targeting proofreading PCR amplification was performed in a volume of 25 µl, containing 5 ng cDNA, 1x Phusion HF Reaction Buffer (New England Biolabs), 10 pmol of each primer, 200 µM dNTPs (Promega, Madison, WI) and 0.5 unit of Phusion Hot Start Flex DNA Polymerase (New England Biolabs). An initial denaturation step at 98 °C for 2 minutes, was followed by 35 cycles of denaturation at 98 °C for 15 seconds, annealing at 65 °C (TRAV) or 60 °C (TRBV) for 30 seconds and extension at 72 °C for 30 seconds. Finally, a 5 minute extension step was performed at 72 °C. Amplicons were purified using Nucleospin Gel and PCR Clean-up (Macherey-Nagel GmbH, Düren, Germany) according to manufacturer's protocol.

Purified amplicons were ligated into the pJET1.2/blunt Cloning Vector (Thermo Scientific) using T4 ligase (Roche Diagnostics GmbH, Mannheim, Germany), and cloned into XL1-Blue Competent Cells. The TRAV12 and TRBV6 constructs from single colonies were isolated using PureYield Plasmid Miniprep System (Promega, Madison, WI) according to manufacturer's protocol. The constructs were sequenced using the T7 promoter target site, TCR sequences were analysed using IMGT/V-QUEST version 3.2.16.

CHAPTER 5

Bioluminescent reporter assay for monitoring ER stress in human beta cells

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ABSTRACT

During type 1 diabetes development, cells in the islets of Langerhans engage adaptive mechanisms in response to inflammatory signals to cope with stress, to restore cellular homeostasis, and to preserve cell function. Disruption of these mechanisms may induce the formation of a repertoire of stress-induced neoantigens, which are critical in the loss of tolerance to beta cells and the development of autoimmunity. While multiple lines of evidence argue for a critical role of the endoplasmic reticulum in these processes, the lack of tools to specifically monitor beta cell stress hampers the development of therapeutic interventions focusing on maintaining endoplasmic reticulum homeostasis. Here we designed and evaluated a stress-induced reporter in which induction of stress correlates with increased light emission. This Gaussia luciferase-based reporter system employs the unconventional cytoplasmic splicing of XBP1 to report ER stress in cells exposed to known ER stress inducers. Linking this reporter to a human beta cell-specific promotor allows tracing ER stress in isolated human beta cells as well as in the EndoC-βH1 cell line. This reporter system represents a valuable tool to assess ER stress in human beta cells and may aid the identification of novel therapeutics that can prevent beta cell stress in human pancreatic islets.

INTRODUCTION

Beta cell destruction in Type 1 diabetes (T1D) results from the combined effect of inflammation and autoimmunity. The presence of endoplasmic reticulum (ER) stress markers during insulitis points to the involvement of an ER stress response in beta cell destruction[1]. The ER is a central organelle for protein synthesis, processing and folding and essential in insulin biosynthesis, maturation and secretion[2, 3]. Perturbations of the ER homeostasis by environmental factors triggers the induction of an unfolded protein response (UPR) and activation of inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and the cleavage of membrane bound activating transcription factor 6 (ATF6). Activation of these ER membrane bound sensors leads to phosphorylation of eukaryotic translation initiation factor 2α by PERK[4], activation of transcription factor X-box binding protein 1 (XBP1) via nonconventional XBP1 RNA splicing by IRE1α[5, 6] and translocation of ATF6 to the nucleus[7], respectively. These different pathways ultimately act in concert to restore ER homeostasis by the coordinated regulation of inhibition of protein synthesis, degradation of aberrant translation products by the ERAD degradation pathway and enhanced protein folding capacity by upregulation of chaperone expression.

The high insulin translation rate makes beta cells extremely sensitive to ER stress[8] and several lines of evidence suggest that the UPR is a key mechanism for the formation of neoantigens and subsequent autoimmune destruction of beta cells[9-11]. We and others have shown that pathophysiological conditions characteristic for T1D participate to the increased complexity of the beta cell proteome by affecting alternative splicing events[12], formation of defective ribosomal products (DRiPs)[13], activation of post translational modification enzymes leading to citrullination and deamidation of autoantigens[14-18]. These processes are likely to increase visibility of beta cells to immune cells and their subsequent destruction. Thus, monitoring and understanding the origin of beta cell stress is critical to understand autoimmunity, to prevent beta cell failure and to design therapeutics to prevent T1D development. While quantitative methods for monitoring ER stress *in vitro* are well established, these methods are labour intensive and cannot be translated to selectively address beta cell stress in human pancreatic islets because of their multi-endocrine nature. In this study, we describe a quantitative bioluminescent method to measure ER

stress by exploiting the UPR-induced IRE1α-mediated splicing of XBP1 coupled to a Gaussia luciferase reporter gene. We show that this reporter accurately reflects the ER stress status in the human beta cell line EndoC-βH1 during inflammation when compared to classical ER stress quantification methods. Furthermore, it can be used to specifically monitor beta cell stress in primary human islets when the reporter expression is driven by the human insulin promoter (HIP). This reporter represents a novel tool to identify therapeutics targeting beta cell stress in a drug screening platform in human beta cells.

RESULTS

Design of ER stress reporter

Activation of the IRE1 endonuclease by ER stress leads to unconventional XBP1 splicing in which a 26 nucleotide intronic region is removed (Fig. 1a,b). This process causes a shift of the reading frame and gives rise to translation of an elongated C-terminal protein as observed by Western blot analysis of lysates from 293T cells exposed to ER stress-inducing agent thapsigargin (TG) (Fig. 1c). We exploited this stress-induced splicing mechanism to generate a lentivirus vector containing a stress-inducible Gaussia luciferase reporter (pLV-CMV-XBP-GLuc-bc-Puro) (Fig. 1d). In this bi-cistronic construct, where the puromycin resistant gene can be used for clone selection, the ER stress-dependent splicing positioned the Gaussia luciferase coding sequence in frame with the XBP1 *bona-fide* AUG to generate a XBP-Gaussia luciferase fusion protein. Following transfection in HEK 293T cells with the XBP-GLuc construct, treatment with TG lead to up to increase XBP1 splicing as detected by mRNA analysis (Fig. 1e) and to 10-fold induction in light emission after 24h treatment, compared to untreated cells, indicating that the reporter is induced by ER stress (Fig. 1f).

Validation and characterization of the ER stress reporter in 293T cells

To test the sensitivity of the stress reporter in more detail, 293T cells were transfected with pLV-CMV-XBP-GLuc and exposed to various amounts of TG for 6 hours or to a moderate amount of stress agent and analysed over a 24-hour period. ER stress was evaluated by i) the conventional ER stress quantification method, qPCR using primers specific for spliced XBP1 (Fig. 2a, b black symbols); and ii) light emission after cell lysis to quantify reporter splicing (Fig. 2a, b red

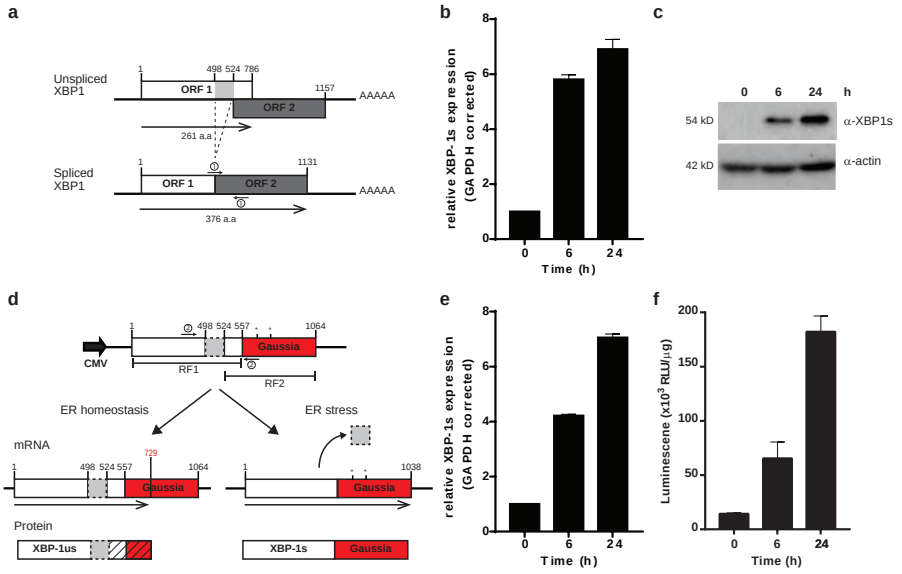

Figure 1. Principle of the ER stress reporter. (a) Schematic representation of XBP1 mRNA under normal (top) and ER stress conditions (bottom). XBP1 mRNA consists of two open reading frames (ORFs) (white and dark grey box). In normal conditions, the retained intron (light grey box) limits translation to the first ORF. Upon ER stress, the intronic region is spliced out by p-IRE1α as indicated by the dashed lines, allowing a shift of the first ORF, to the second ORF generating a protein with an extended C-terminus. The functional ORF is indicated by the black arrow underneath. Position of the primer set for analysis of spliced XBP1 are indicated with small arrows with the number 1. (b) Quantification of XBP1s by qPCR in 293T cells after exposure to TG for 0, 6 or 24 hours. Quantitative PCR analysis was performed in triplicate and GAPDH corrected. (c) Western blot analysis of 293T cell lysates treated with 100 nM TG for 0, 6 or 24 hours analysed with an anti-XBP1-spliced antibody (upper panel) and anti-actin antibody (lower panel). (d) Schematic representation of the ER stress reporter construct, pLV-CMV-XBP-GLuc-bc-Puro, encoding XBP1 (white box) with retained intron (light grey box) and Gaussia luciferase (red box) driven by a CMV promoter. The two ORFs are indicated by black lines. In normal conditions, the intronic region is retained and only the first ORF is translated (left arm). The splicing of XBP1 during ER stress results in the translation of the first and second ORF resulting in an XBP1-Gaussia fusion protein (right arm). In red the in-frame STOP codon of the first ORF is depicted. The diagonal line pattern indicates the translational product is not in the functional reading frame. Position of the primer set for analysis of reporter splicing are indicated with small arrows with the number 2. (e) Quantification of XBP1s by qPCR in cells transfected with the reporter construct after exposure to TG for 0, 6 or 24 hours. Quantitative PCR analysis was performed in triplicate and GAPDH corrected. (f) Luciferase activity measured in pLV-CMV-XBP-GLuc-bc-Puro transfected 293T cell lysates priorly treated with 100 nM TG for 0, 6 or 24 hours. Results are shown as relative light units (RLU) corrected by the protein amount in the sample.

symbols). In these experiments, a similar spectrum of ER stress quantification was obtained with both ER stress quantification methods. Using both techniques we show that XBP1 splicing is occurring at minimal amounts of TG, shortly after exposure to stress inducer and rapidly reaching a plateau when higher doses are used or 8 hours post stimulation. Light emission correlated

strongly with the splicing of the reporter construct as determined by PCR using primers specific for the reporter (Fig. 2a, b lower panels). To evaluate whether the reporter construct may be used as drug screening assay, we tested the effect of the MKC3946[19], an inhibitor of inositol-requiring enzyme 1α in HEK293T cells in presence of ER stress inducer TG. As anticipated, the induction of stress by TG lead to a substantial increase in XBP1s RNA expression. This effect was countered by the presence of the MKC3946, indicating effective inhibition of XBP1 splicing (Fig. 2c). Similar results were obtained by measuring Gaussia luciferase activity in the cell lysates (Fig. 2d).

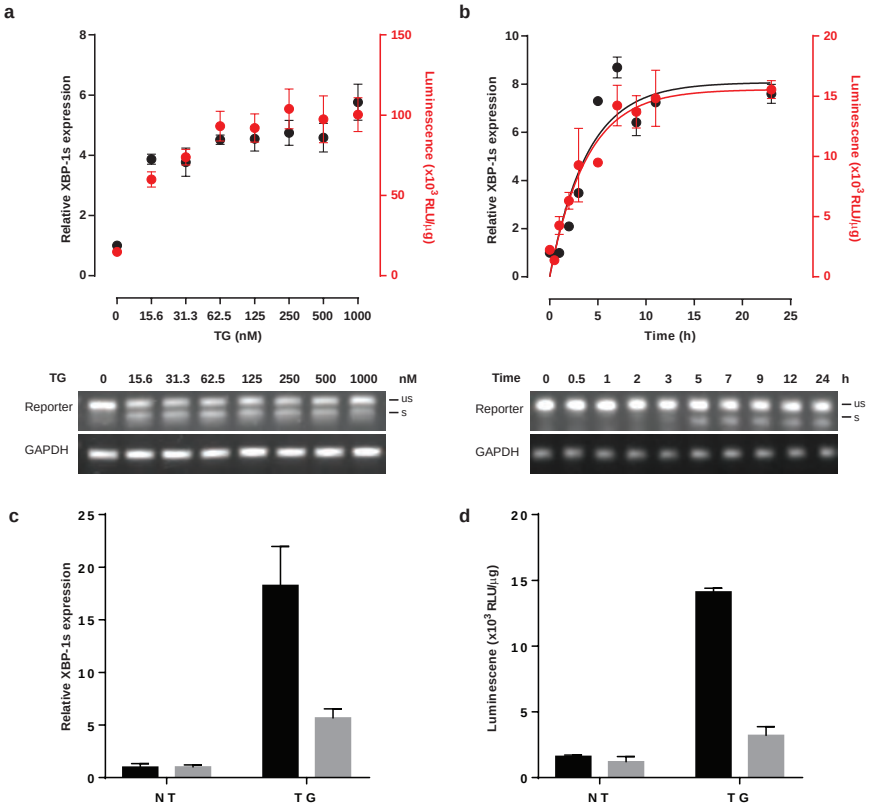

Figure 2. Characterization of pLV-CMV-XBP-GLuc-bc-Puro in 293T cells. (a) Analysis of the ER stress-induced reporter in transfected 293T cells exposed to various amounts of TG by classic validation of endogenous XBP-1 splicing by qPCR (black dots) and luciferase activity (red dots) (upper panel); Validation of reporter splicing by PCR analysis of reporter and GAPDH (lower panel) on agarose gel. (b) Similarly, a time course analysis of the ER stress reporter in transfected 293T cells stimulated with 50 nM TG was performed. Quantitative PCR analysis was performed in triplicate and GAPDH corrected. Gaussia activity is expressed in relative light units per µg protein. Each point represents the average of 3 experimental replicates. Data are shown as the mean ± SEM. (c) qPCR analysis of the level of

expression of XBP1s in 293T cells transfected with the reporter construct after 24 hours treatment with 100 nM TG in presence (grey bars) or absence (black bars) of 5 μM MKC3946. Quantitative PCR analysis was performed in triplicate and GAPDH corrected. (d) Luciferase activity measured in transfected 293T cell lysates after 24 hours treatment with 100 nM TG in presence (grey bars) or absence (black bars) of 5 μM MKC3946. Results are shown as relative light units (RLU) corrected by the protein amount in the sample.

Altogether, these data indicate that the reporter accurately reflects stress-induced splicing of endogenous XBP-1, even shortly after exposure to low amount of stress inducing agent and allows screening of ER stress inhibitor drugs.

Evaluation of the ER stress reporter in human beta cells

Beta cell stress is a significant component underlying the pathogenesis of T1D. Recently, the human EndoC-βH1 beta cell line has been generated as a promising model to study human beta cell biology under T1D pathophysiological conditions[20]. In order to evaluate the potential of the XBP-GLuc construct to detect ER stress in a relevant beta cell model, we generated a stable reporter cell line by lentiviral transduction followed by puromycin selection; pLV-CMV-GFP-bc-Puro transduced cells were used as control (Fig. 3a). We confirmed that lentiviral transduction and expression of the reporter construct had no apparent impact on beta cell viability (Fig. 3b) and function as seen by c-peptide secretion after glucose challenge (Fig. 3c). Moreover, the presence of the transgene had no effect on the baseline of UPR related gene expression and capacity to respond to stress stimulation as seen by the increased expression of ATF3 and CHOP upon cytokines stimulation (Fig. 3d). The modified cells were then exposed to proinflammatory cytokines mimicking the pathogenic inflammatory beta cell microenvironment of pancreatic islet in type 1 diabetes, i.e. IFNγ/IL1β or IFNγ/IL1β/TNFα and analysed for endogenous XBP1 splicing by qPCR or light emission in cell lysates. Both treatments led to the generation of endogenous XBP1s, which correlated with an increase in luminescence (Fig. 3e). Of note, the induction of stress is enhanced by the presence of TNFα in the cytokine mixture.

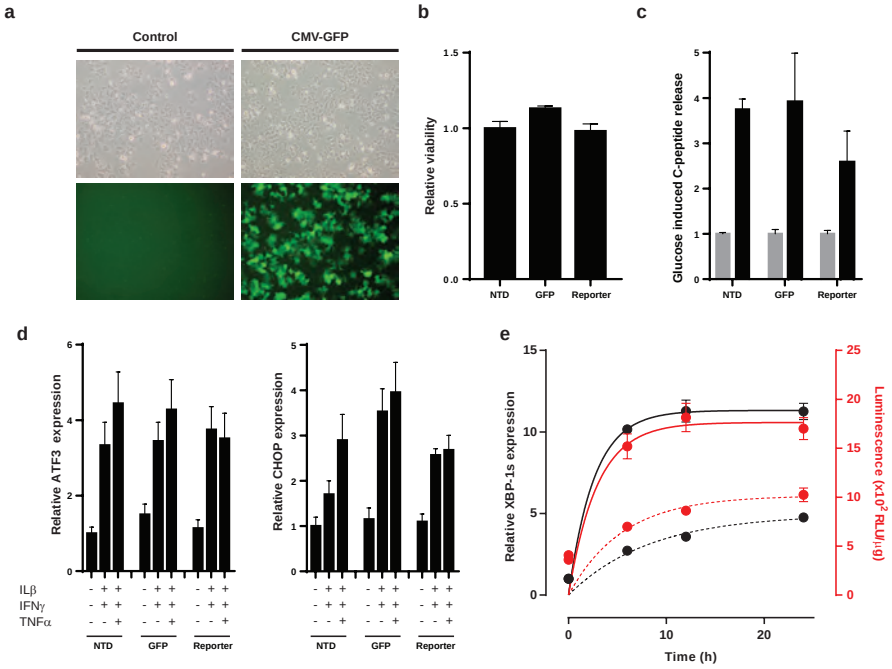

Figure 3. Evaluation of the ER stress reporter in human beta cells. (a) Transduction of EndoC-βH1 with LV-CMV-GFP after puromycin selection (right panels) compared to non-transduced cells (left panels). (b) Cell viability as determined by WST assay in non-transduced cells (NTD), GFP transduced (GFP) or transduced with the pLV-CMV-XBP-GLuc-bc-Puro construct (reporter). Data are shown as relative viability compared to non-transduced cells (set to 1). (c) C-peptide release assay performed on non-transduced cells (NTD), GFP transduced (GFP) or the reporter cell lines (reporter) after low glucose stimulation (grey bars) or high glucose stimulation (black bars). Data are shown as induction c-peptide release, low glucose conditions set to 1. (d) qPCR analysis of the level of expression of ATF3 and CHOP in non-transduced cells (NTD), GFP transduced (GFP) or the reporter cell lines (reporter) after cytokines treatment. Quantitative PCR analysis was performed in triplicate and GAPDH corrected. The data are shown as relative expression. The expression in non-transduced cells in absence of stimulation is set to 1 and used as reference. (e) Analysis of the reporter EndoC-βH1 cells exposed to 1000 U/ml IFNγ and 2 ng/ml IL-1β (dashed lines) and 1000 U/ml IFNγ, 2ng/ml IL-1β and 50 ng/ml TNFα (solid lines) for 0, 6, 12 and 24 hours. ER stress was validated using endogenous XBP-1 splicing by qPCR (black lines and dots) and luciferase activity (red lines and dots).

While classical methods allow a precise examination of the ER stress status, the presence of various cell types in the islets of Langerhans hampers the use of these methods for specific investigation of beta cells. In order to study beta cell stress within their islet environment we replaced the CMV promoter by the human insulin promoter (HIP) to drive expression of the transgene specifically in insulin-producing cells (Fig. 4a)[21]. We show that while lentivirus vectors can moderately modify endocrine cells (~30% transduction efficiency) (Fig. 4b), the

use of the human insulin promoter restricts expression of the transgene to beta cells (Fig. 4c).

Following transduction with the pLV-HIP-XBP-GLuc-bc-GFP lentiviral vector, human endocrine cells were exposed to pro-inflammatory cytokines. Using this assay, we demonstrate that primary human beta cells respond to inflammatory cytokines by eliciting an ER stress response, as reflected by the increased Gaussia activity. Furthermore, our data point to a major contribution of IFNγ/IL1β in this process since no increase in light emission was detected upon addition of TNFα to the cytokine mixture (Fig. 4d).

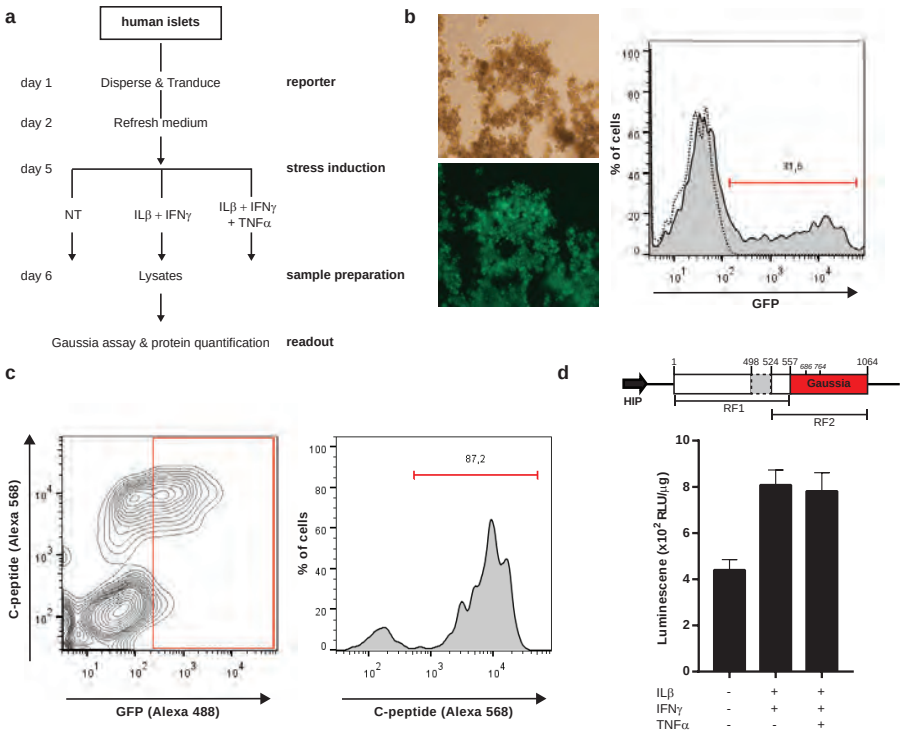

Figure 4. Evaluation of the ER stress reporter in primary human beta cells (a) Flow diagram of the experimental design for the evaluation of beta cell stress in human pancreatic islets. (b) Flow cytometry analysis showing the transduction rate of primary human islet cells after cell dispersion and lentivirus transduction with LV-CMV-GFP (MOI=3). (c) Flow cytometry analysis of primary human islets cells stained with anti-c-peptide and anti-GFP antibodies after cell dispersion and transduction with LV-HIP-GFP (MOI=3). (d) Schematic representation of the beta cell-specific ER stress reporter construct, encoding XBP1 (white box) with retained intron (light grey box) and Gaussia luciferase (yellow box) driven by a HIP promoter (black box arrow), and underneath the two reading frames (indicated by black lines) (top). Graph depicts the luminescence measured in lysates made of transduced human islets after exposure to inflammatory cytokines (1000 U/ml IFNγ, 2ng/ml IL-1β and 50 ng/ml TNFα) (bottom). Each point represents the average of 3 experimental replicates. Data are shown as the mean ± SEM.

DISCUSSION

Islets of Langerhans are responsible for maintaining glucose homeostasis. The ER forms the integration interface between the cellular microenvironment and adaptation. Dysfunction of this interface may contribute to the development of T1D by generation of neoantigens targeted by the immune system and by inducing cell death pathways. Therefore, the ability to monitor ER stress is an essential step in understanding disease pathogenesis and for the development of therapeutic intervention.

The Gaussia luciferase reporter assay presented here is an easy and rapid alternative to traditional methods. In contrast to previous approaches, the stress status can be directly measured in cell lysates upon addition of substrate, without the need of laborious RNA isolation or protein analysis procedures. The IRE1α-XBP1 pathway has a critical role in life and death decision by preserving ER homeostasis versus triggering apoptosis by activating the regulated IRE1-dependent decay (RIDD)[22]. In beta cells, the IRE1α-XBP1 pathway participates to the maintenance of the balance between the beta cell microenvironment and beta cell function as it is required for proper insulin biosynthesis and glucose responsiveness[23-25]. By exploiting XBP1 processing in the reporter construct, we demonstrate that this reporter accurately reflects endogenous XBP-1 splicing after both chemical- and cytokines-induced stress in 293T and EndoC-βH1 cells. This approach appeared equally sensitive as currently used ER stress detection methods. The generation of stable EndoC-βH1 cells expressing the ER stress reporter set the stage for future high throughput compound screening aiming at relapsing ER stress[20] and can be combined with other assays to monitor beta cell functionality, such as the glucose stimulated insulin secretion (GSIS) assay.

The multi-endocrine nature of human pancreatic islets often complicates the analysis of beta cell stress in primary islets, as the classical methods do not distinguish which cells are stressed but rather provide a general picture of stressed islet cells as whole. This impairs assessing beta cell stress selectively, as the other endocrine cell may respond differently to changes in the microenvironment counteracting the beta cell stress[26, 27]. Single cell analyses of pancreatic islets are currently the only method offering a real reflection of beta cell stress specifically. Yet, these techniques are difficultly translatable for drugs screening approach. The method described here, using the human insulin promoter to drive transgene expression of an ER stress reporter specifically

in beta cells, represents a rapid and robust alternative allowing the specific analyses of beta cell ER stress within the total islet environment. Our results using the IRE1α inhibitor, on HEK293T cells show the potential application of the reporter as drug screening platform.

Our results indicate that EndoC-βH1 cells respond differently to cytokines when compared to human islets. The relative low induction of ER stress detected in primary beta cells compared to the beta cell line after cytokine stimulation, might be the result of a high basal level of ER stress in islets resulting from islet isolation, *in vitro* culture conditions and cell dispersion thereby dampening *in vitro* ER stress responses to cytokines. Moreover, EndoC-βH1 cells seem particularly sensitive to TNFα in the cytokine stimulation cocktail. Previous studies comparing the response of the human beta cell line and primary islets to cytokines, have shown that the EndoC-βH1 cells may have an impaired ER stress response and do not faithfully reflect the response of human islets to cytokines[28]. While differences in response to cytokines between EndoC-βH1 and primary human beta cells could reflect intrinsic differences between the cell line and primary cells (e.g. EndoC-βH1 cells divide while primary beta cells do not), these differences could also point towards a guardian role of other endocrine cells[27]. The glucagon like peptide 1 (GLP1) produced by alpha cells for example could represent a possible paracrine mediator of this anti-inflammatory effect[29]. Therefore, caution should be taken with the use of beta cell lines and, where feasible, results obtained from cell lines should be confirmed in primary human beta cells.

While similar strategies for ER stress evaluation have been successfully used in transgenic mice with a XBP1-GFP variant[30], the combination of the lentivirus vector that can efficiently modify cells without affecting cell function[31] and the Gaussia luciferase allow a sensitive and quantitative method to measure stress. We anticipate that this reporter used in combination with the human insulin promoter will represent a powerful tool to address various issues regarding ER stress in beta cells in type 1 diabetes as well as type 2 diabetes.

MATERIALS AND METHODS

Cell culture

HEK293T cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL), 100 units/ml Penicillin and 100 µg/ml streptomycin (Gibco-BRL).

The human beta cell line, EndoC-βH1 cells[32], was obtained from Dr. Raphael Scharfmann (Paris Descartes University, France) and maintained in low glucose DMEM supplemented with 5.5 µg/ml human transferrin, 10 mM nicotinamide, 6.7 ng/ml Selenit, 50 µM β-mercaptoethanol, 2% bovine serum albumin fraction V, 100 units/ml Penicillin and 100 µg/ml streptomycin. Cells were seeded in ECM, fibronectin pre-coated culture plates. Inflammatory stress was induced by treating EndoC-βH1 with a mixture of 1000 U/ml IFNγ, 2 ng/ml IL1β and 50 ng/ml TNFα for the indicated time.

Transfection

HEK293T were transfected in suspension using polyethylenimine (PEI). Transfection mixes, consisting of 0.125 µg plasmid DNA, 0.375 µg/µl PEI in a total volume of 12.5 µl Opti-MEM (GIBCO) for a 96-well, were incubated for 10 minutes at room temperature prior adding to the cell suspension. After 24 hours cells were treated with Thapsigargin (Bio-connect). The concentrations and incubation times are indicated in the figures. For treatment with the IRE1α inhibitor, transfected cells were pre-treated for 4 h with MKC3946 (5 µM) before addition of 0.1 µM Thapsigargin for 24 hours.

Human islet isolation and culture

Pancreatic islets were obtained from human organ donor pancreata. Human islets were isolated from organ donors. Islets were only studied if they could not be used for clinical purposes and if research consent had been obtained. According to the national law ethics approval is not required for research on donor tissues that cannot be used for clinical transplantation. The isolations were performed in the GMP-facility of LUMC according to the previously described protocol[33]. For experimental use, human islets were maintained in ultra-low attachment plates (Corning, NY 14831) in low glucose DMEM supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml streptomycin. Dispersed

islet cells were treated with 1000 U/ml IFNγ, 2 ng/ml IL1β and 50 ng/ml TNFα for 24 hours to induce ER stress. All methods were carried out in accordance with relevant guidelines and regulations.

Lentivirus production and transduction

Lentiviruses were produced as previously described[31]. Prior transduction the human pancreatic islets were dispersed, then cocultured with virus supernatant supplemented with 8 µg/ml polybrene (Sigma). After overnight incubation, the medium was refreshed and the cells were allowed to rest for 3 days, before continuing with other treatments and analysis. EndoC-βH1 stably expressing the reporter construct were established after transduction with the pLV-CMV-XBP-GLuc-bc-Puro lentivirus (MOI=5) and puromycin selection (1 µg/ml).

Human islets cells have been transduced at MOI=3 immediately after cell dispersion by trypsin as previously described[13, 21]. After overnight incubation, the medium was refreshed and dispersed islet cells were maintained in culture in ultra-low attachment plate (Corning, NY 14831) for the duration of the experiment.

Construction of a beta cell-specific, stress-induced reporter

The XBP-Gaussia fragment contains the first 558 nucleotides of XBP1 coding sequence fused to the last 504 nucleotides from the GLucM43I encoding sequence[34]. The DNA fragment was synthesized by Geneart (thermofisher, Regensburg Germany). pLV-CMV-XBP-GLuc-bc-Puro was generated by insertion of the XBP-Gaussia fragment into pLV-CMV vector. The XBP Gaussia fragment was then subcloned into the pLV-HIP vector [21] to generate a pLV-HIP-XBP-GLuc-bc-GFP vector.

RNA isolation and quantitative PCR (qPCR)

RNA was extracted using TRIzol reagent (invitrogen) according to manufacturer's procedures. To avoid DNA contamination samples were DNase treated prior cDNA synthesis. RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). SybrGreen master mix kit (Applied Biosystems) was used for detection of endogenous XBP1 splicing, ATF3, CHOP and GAPDH by qPCR with the following primers: XBP1s Fw 5'-CTGAGTCCGCAGCAGGTG-3', XBP1s Rv 5'-GAGATGTTCTGGAGGGGTGA-3'; ATF3 Fw 5'-GTGCCGAAACAAGAAGAAGG-3', ATF3 Rv

5′-TCTGAGCCTTCAGTTCAGCA-3′; CHOP Fw 5′- GACCTGCAAGAGGTCCTGTC-3′, CHOP Rv 5′- CTCCTCCTCAGTCAGCCAAG-3′; GAPDH Fw 5′-ACAGTCAGCCGCATCTTCTT-3′, GAPDH Rv 5′-AATGAAGGGGTCATTGATGG-3′. All measurements were performed in triplicate and GAPDH was used for normalization. The detection of reporter splicing was assessed by PCR using the following primers: Reporter Fw 5′-GAAGCCAAGGGGAATGAAG-3′, Reporter Rv 5′-CCGTGGTCGCGAAGTTGCTGG-3′. PCR reactions were analysed on 2% agarose gel.

Western blot analysis

Cells were lysed in Tropix lysis mix (Applied Biosystems). Total protein content of the lysates was determined using Bradford reagent assay (Biorad, Veenendaal, The Netherlands). Samples were boiled with sample buffer (10% glycerol, 2% SDS, 50 mM Tris-HCl pH 6.8, 0.1% Bromophenol blue and 1% β-mercaptoethanol) and 50 µg protein was loaded per well of a 10% SDS-polyacrylamide gel. The proteins were subsequently transferred to Immobilon-P (Millipore, Etten-Leur, the Netherlands) and visualized by standard protocols with rabbit-anti-XBP1, specific for the spliced XBP1 form (1:1000; Biolegend POLY6195) and mouse-anti-actin (1:2000 C4; Millipore) and corresponding HRP-conjugated antibodies goat-anti-rabbit IgG (1:5000 sc-2004, Santa Cruz Biotechnology) and goat-anti-mouse IgG HRP (1:5000 sc-2005, Santa Cruz Biotechnology).

Gaussia activity measurement

Gaussia activity was determined in cell lysates by luminometry after addition of 1 mM coelenterazine (Promega). Experiments were performed in triplicate for every condition. Results are corrected for the lysate protein content and represented as average Relative light units (RLU) per µg protein.

Glucose induced C-peptide assay

EndoC-βH1 were seeded 50,000 cells per well in a 96 well plate. Two days post plating, cells were preincubated in a modified Krebs-Ringer Bicarbonate buffer (KRBH) containing 115 mmol/l NaCl, 5 mmol/l KCl, 24 mmol/l NaHCO3, 2.2 mmol/l CaCl2, 1 mmol/l MgCl2, 20 mmol/l HEPES, 2 g/l human serum albumin (Cealb, Sanquin, The Netherlands), pH 7.4 for 2 hours. After preincubation, the buffer was successively changed for 1 hour to KRBH with 2 mmol/l and 25 mmol/l glucose at 37 °C. C-peptide concentrations were determined in the supernatants by ELISA (10-1136-01 Mercodia, Uppsala, Sweden).

C-peptide staining

Following dispersion, single cell islet cells were fixed and permeabilized in PBS containing 4% paraformaldehyde and 0,1% saponin. Blocking was done with 1%BSA,0,1% saponin in PBS and in which primary and secondary antibodies were also diluted. Primary antibodies against C-peptide (1:1000; Millipore CBL94) and GFP (1:1000) and secondary antibodies coupled to Alexa 568 (1:500) and Alexa 488 (1:500) were used.

Viability assay

Cell viability was assessed by replacing the culture medium by fresh medium containing 10% WST-1 reagent (Roche, Penzburg, Germany). The cells were maintained in the incubator at 37 ^{0}C, 5% CO_2 for 3 h before the absorbance was measured in a microplate reader (Bio-Rad model 550, Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm. The results are shown as relative viability compared to non-transduced cells.

Statistical analysis

Data are presented as mean ± SEM. Trend lines were generated by nonlinear regression analysis following a one phase exponential association equation. Calculations were performed using GraphPad Prism 7.

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Competing interest

The authors declare no competing interests.

Author Contributions

M.J.L.K performed the experiments and wrote the manuscript, E.J.P.K provided with human islets and wrote manuscript; R.C.H., B.O.R., A.Z. supervised the project, designed the experiments and wrote the manuscript.

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CHAPTER 6

Expression of alternative insulin gene-derived proteins in human pancreatic islets

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ABSTRACT

Blood glucose homeostasis is tightly regulated by the endocrine cells within the islet of Langerhans. Protein Expression of insulin, glucagon, and somatostatin is restricted to beta, alpha, and delta cells, respectively. Yet, recent transcriptome analyses have revealed that insulin mRNA is also detected in non-beta endocrine cells. We studied alternative splicing of human insulin mRNA in pancreatic islets and identified the presence of an insulin mRNA isoform. This transcript, encoding the complete preproinsulin signal peptide and B-chain, yields an altered C-terminus due to the disruption of the original reading frame. The resulting polypeptide is largely identical with the previously identified insulin-derived defective ribosomal protein (INS-DRiP). Immunohistochemistry on pancreatic sections using antisera raised to detect these alternative insulin gene-derived products shows the presence of INS-DRiP in insulin-producing beta cells and the product of a splice variant primarily in somatostatin-producing delta cells. Although the role of this insulin splice variant has not yet been defined, its presence in secretory granules of somatostatin-positive cells, as revealed by electron microscopy, suggests an endocrine or paracrine function.

INTRODUCTION

While polyhormonal endocrine cells have been shown to reside in human foetal pancreatic islets and in individuals with chronic pancreatitis, fully differentiated endocrine cells are classically dedicated to producing a single hormone; i.e. glucagon, insulin, somatostatin production by alpha, beta, and delta cells, respectively[1-3]. Under this definition, insulin gene expression is restricted to the pancreatic beta cells. However, accumulating data indicate mature human beta cells are more plastic than previously assumed[4]. Whereas the differentiated state is maintained by reinforcement of beta cell-specific gene regulatory networks and repression of other transcriptional programs[5-8], metabolic and mechanical stress have been shown to cause spontaneous dedifferentiation and transdifferentiation of human beta cells. Conversion of beta cells to alpha and delta cell-like states observed in T2D patients has been proposed to cause beta cell failure[9]. In addition, *in vitro* reaggregation of human islet cells induced spontaneous beta cell conversion into glucagon-producing cells[10]. This endocrine plasticity has been proposed to allow dysfunctional beta cells to escape apoptosis due to environmental stress as well as replenish the beta cell population[11, 12]. In situ hybridisation and single-cell transcriptome analysis of human islet cells have confirmed the presence of insulin mRNA in alpha and delta cells[13, 14]. Approximately 46% of islets cells were found to express more than one hormonal transcript type per cell, with a considerable portion of endocrine cells containing both insulin and somatostatin transcripts[15].

Alternative splicing increases proteome diversity by generation of multiple mRNA transcripts from a single gene that differ in assembly of exons and introns. It is estimated that approximately 95% of the human transcriptome is alternatively spliced[16]. Tissue-specific splicing patterns allow expression of similar genes in different cell types to produce different proteins which differ in biologic composition and activity[17]. Alternative splicing networks are implicated in a broad variety of biological processes, including: maintenance of pluripotency, directing cell differentiation, and cell lineage commitment[18-21]. Furthermore, splicing patterns are highly dynamic and therefore provide a mechanism to swiftly adapt to changes in the local microenvironment[22, 23]. The beta cell transcriptome was shown to be highly impacted by inflammatory and metabolic insults[24, 25]. Experiments conducted in HEK293T cells overexpressing the human insulin gene demonstrated the presence of cryptic splice sites in insulin, as multiple insulin isoforms were detected[26, 27].

In this study, we investigated insulin gene splicing in human pancreatic islets. We generated antisera directed against these alternative insulin gene products and demonstrate that an insulin isoform protein can be detected in delta cells. More specifically, high resolution electron microscopy and immune-labelling revealed that this variant insulin gene product localized to the secretory granules.

RESULTS

Evidence of alternative insulin RNA splicing in human islets

Human insulin is encoded by a small gene with only three exons and two introns. Yet, PCR analyses performed on RNA isolated from human islets of three different donors show the presence of two insulin transcript isoforms (Fig. 1A). Nucleotide sequencing of the PCR fragments indicated that the larger, most abundant isoform represents wild-type insulin (INS-wt) in which intron 1 and 2 have been fully spliced out. We recently showed that this mRNA, known to encode preproinsulin (PPI), generates a defective ribosomal product (DRiP) that is target of islet autoimmunity and associated with type 1 diabetes (T1D) pathology[28]. The shorter isoform results from a cryptic splicing site within exon 3 at position 1338, predicted from *in silico* analysis (supplementary Fig. 1). The altered open reading frame that is formed by alternative splicing may lead to the generation of a polypeptide during translation in which the signal peptide and B-chain of the canonical PPI are intact but the C-terminal end of the molecule is different (referred to as INS-splice). Coincidently this C-terminal region would be identical to the INS-DRiP with except of the first 10 N-terminal amino acids that contains the diabetogenic T cell epitope (Fig. 1B)[28].

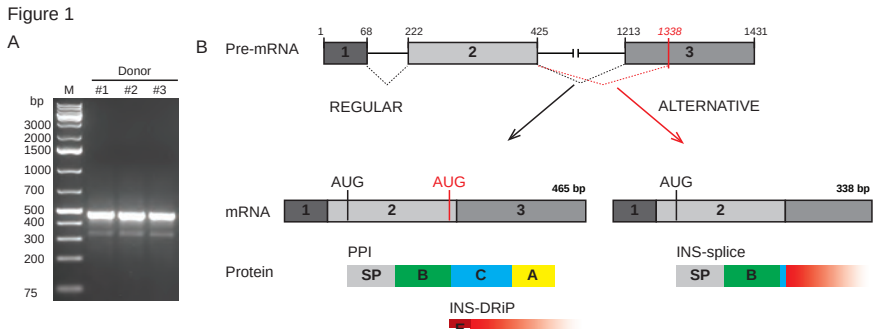

Figure 1: Insulin gene-derived products. A) Analysis of insulin gene derived transcript splicing by PCR on RNA derived from human pancreatic islets of 3 different donors (#1, 2 and 3) visualized on a DNA gel. The DNA marker is indicated by the M. B) Schematic overview of

the human insulin pre-mRNA with the exons annotated by numbers (1-3) and the intronic regions represented by a black solid line. Normal insulin splicing and alternative insulin splicing are indicated by black and red dashed lines, respectively. The resulting mRNA products with translation initiation sites are depicted underneath. For each mRNA molecule the potential protein products are displayed, including preproinsulin (PPI) with the signal peptide (SP, grey), B-chain (B, green), C-peptide (C, blue), and A-chain (A, yellow) specified. In red the INS-DRiP with the E, indicating the previously identified CD8 T-cell epitope and an insulin splice variant (also known as INS-splice). Corresponding amino acid sequences are indicated with corresponding colours, letters indicate the presence of the complete chain.

Alternatively spliced insulin is a template for translation in human islets

In order to investigate these alternative insulin gene-derived proteins, rabbits were immunized with a short synthetic polypeptide unique to INS-DRIP (DRiP1-13) and a short synthetic polypeptide of the C-terminus common to both predicted alternative insulin gene-derived products INS-DRiP and INS-splice (FS-c). The peptides were selected from analysis of the UniProt human protein knowledgebase using the basic local alignment search tool (BLAST) to avoid cross reactivity to other known proteins (data not shown). Serum specificity was confirmed by ELISA using recombinant PPI, INS-DRiP and INS-splice. As expected, neither sera cross reacted with PPI (Fig. 2) and while the anti-DRiP1-13 serum specifically detected the INS-DRiP polypeptide, the anti-FS-c antiserum recognized both alternative INS-derived recombinant proteins (Supplementary Fig. 2).

To investigate whether the insulin gene-derived polypeptides are generated by islets, human pancreatic sections were stained with either the pre-immunization or post-immunization antisera. The localisation of the N-terminal INS-DRiP polypeptide within beta cells is in line with our previous findings and supports beta cell destruction by CTL directed against the DRiP (Fig. 3A). Yet, the antiserum raised to the C-terminus common to INS-DRiP and INS-splice did not colocalize with insulin, indicating FS-c positive (FS-c^+) cells are not beta cells (Fig. 3B). To assess the identity of these FS-c^+ cells, human pancreatic sections were analysed for various endocrine cell markers (i.e. insulin, glucagon, and somatostatin). Our results show that the staining of the FS-c epitope was restricted to delta cells as indicated by its colocalization with somatostatin (Fig. 3C). Of note, staining of other endocrine tissues demonstrates that the expression of the INS-splice polypeptide was specific to pancreatic islets (Supplementary Fig. 3).

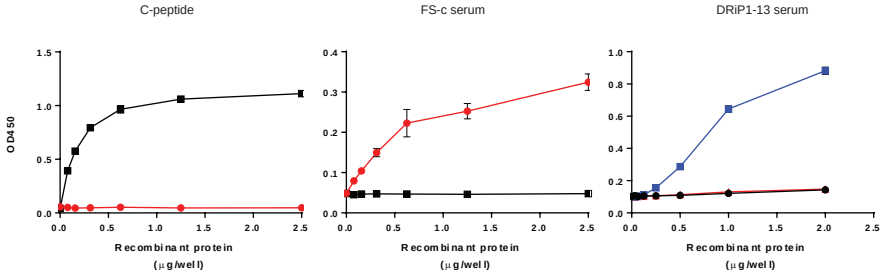

Figure 2: Validation of DRiP1-13 and FS-c antisera. Validation of serum responses in an indirect ELISA assay to serial dilutions of recombinant PPI (black curves) and recombinant INS-splice (red curve) and INS-DRiP (blue) using c-peptide antibody (left panel), FS-c antiserum (middle panel) and DRiP1-13 antiserum (right panel).

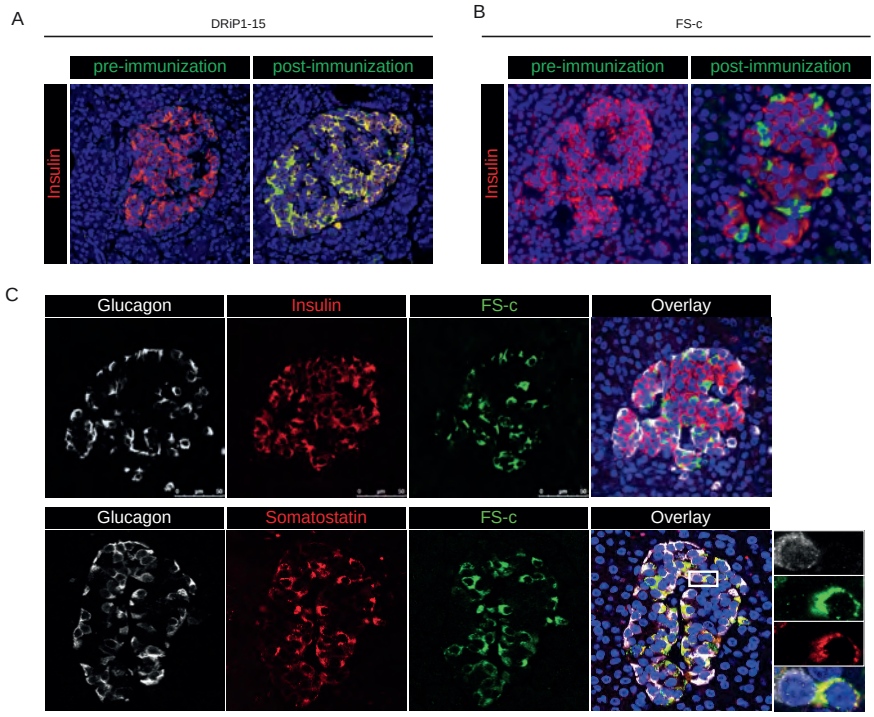

Figure 3: FS-c antiserum exclusively labelled somatostatin-producing delta cells. Immunostaining of human pancreatic sections with pre-immunization serum (left panel) and post-immunization serum (right panel) (green) in combination with insulin (red). A) Serum derived from DRiP1-13 immunized mice. B) Serum derived from FS-c immunized mice. C) Human pancreatic sections stained for glucagon (white) and insulin (red) (middle panels) and glucagon (white) and somatostatin (red) (lower panels) in combination with FS-c antiserum (green). At the lower right enlarged images of the white enclosure are depicted. Nuclei are visualized by DAPI staining (blue).

The FS-c antiserum does not cross react with somatostatin

To validate the presence of an insulin gene-derived product and to exclude cross reactivity with somatostatin, we generated 293T cells expressing the human insulin gene. In these cells, expression of the gene leads to expression of two insulin transcript variants, as observed in human islets (Supplementary Fig. 4A). Western blot analysis of the cell lysates of surrogate beta cells, indicated that PPI is expressed, as well as an alternative insulin-derived product (Fig. 4A). To confirm that the spliced isoform is detected with the antiserum, both transcript isoforms were isolated and cloned into different expression plasmids. Western blot analysis of lysates of 293T cells transfected with the WT insulin cDNA or the spliced cDNA demonstrates that the serum specifically detects the spliced isoform. The C-peptide is only detected in lysates of cells transfected with the INS-wt (Fig. 4B, supplementary Fig. 4B).

Cross reactivity of the serum to delta cell-specific protein somatostatin was excluded by FS-c specificity tested on recombinant somatostatin (Fig. 4C). Furthermore, antibody blocking assays using recombinant somatostatin did not alter detection of recombinant INS-splice, while antibody blocking with recombinant INS-splice markedly reduced INS-splice detection (Fig. 4D). Similarly, antibody blocking using immunization peptide reduced the mean fluorescence of the FS-c^+ islet cell population compared to irrelevant peptide (Fig. 5A). RNA isolation from the FS-c^+ population showed an enrichment of somatostatin and INS-splice RNA in these cells when compared to the FS-c^- cells, confirming our previous finding. (Fig. 5B, Supplementary Fig.4C).

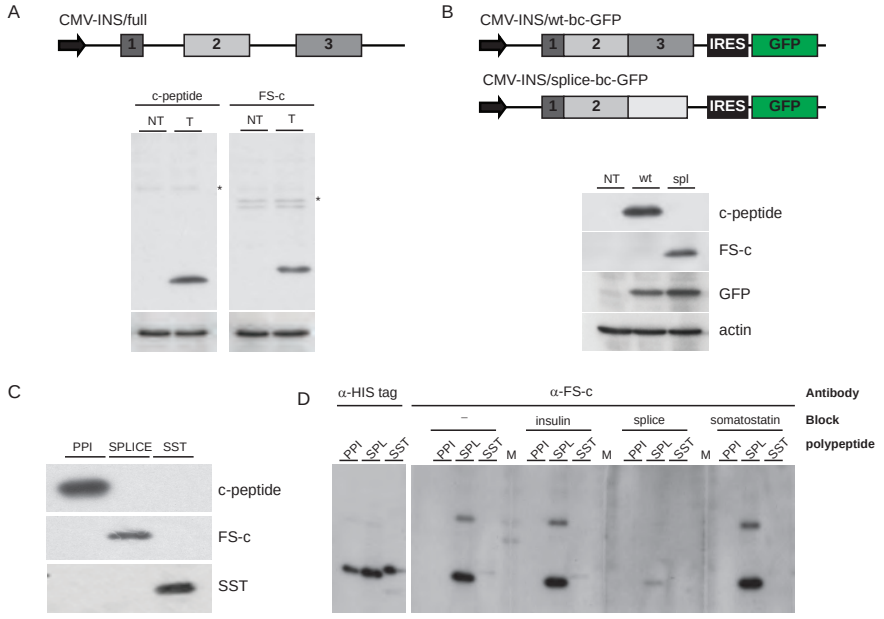

Figure 4: INS-splice is insulin gene-derived and serves as template for translation in 293T cells. A) Schematic representation of the plasmids CMV-INS/full, encoding all exons (annotated by numbers 1-3) and introns (represented by the solid black line) of insulin driven by a CMV promotor. Western blot analysis of 293T cell lysates transfected with CMV-insulin/full (T) and non-transfected cells (NT). Lysates were made 48 hours post transfection and analysed with anti-c-peptide (left panel), anti-FS-c (right panel) and anti-actin (bottom panels). Unspecific background bands (*). B) Schematic representation of CMV-INS/wt-bc-GFP, encoding the mRNA of normally spliced insulin, and CMV-INS/splice-bc-GFP, encoding the mRNA of alternatively splicing insulin in exon 3. With underneath the western blot analysis of 293T cell lysates transfected with CMV-INS/WT-bc-GFP (wt) and CMV-INS/SPLICE-bc-GFP (spl) or non-transfected (NT). Lysates were made 48 hours post transfection and analysed with anti-c peptide (first panel), anti-FS-c (second panel), anti-GFP (third panel) and anti-actin (fourth panel). C) Western blot analysis of recombinant PPI (PPI), recombinant INS-splice (SPLICE) and recombinant somatostatin (SST) with c-peptide antibody (upper panel), FS-c antiserum (middle panel) and somatostatin antibody (lower panel). D) Western blot analysis of recombinant polypeptides with FS-c antiserum that has previously been blocked overnight with recombinant PPI, INS-splice or somatostatin. 15% SDS-polyacrylamide gels were loaded with 50 ng recombinant polypeptide. Antibody blockings were performed with 5 µg recombinant polypeptide. All membranes were developed simultaneously on a single film to exclude differences in band intensity due to differences in exposure time. The M indicate the position of the protein marker were membranes were cut after blotting and put together prior development. Loading of recombinant polypeptides was demonstrated by the presence of the HIS-tag (right panel).

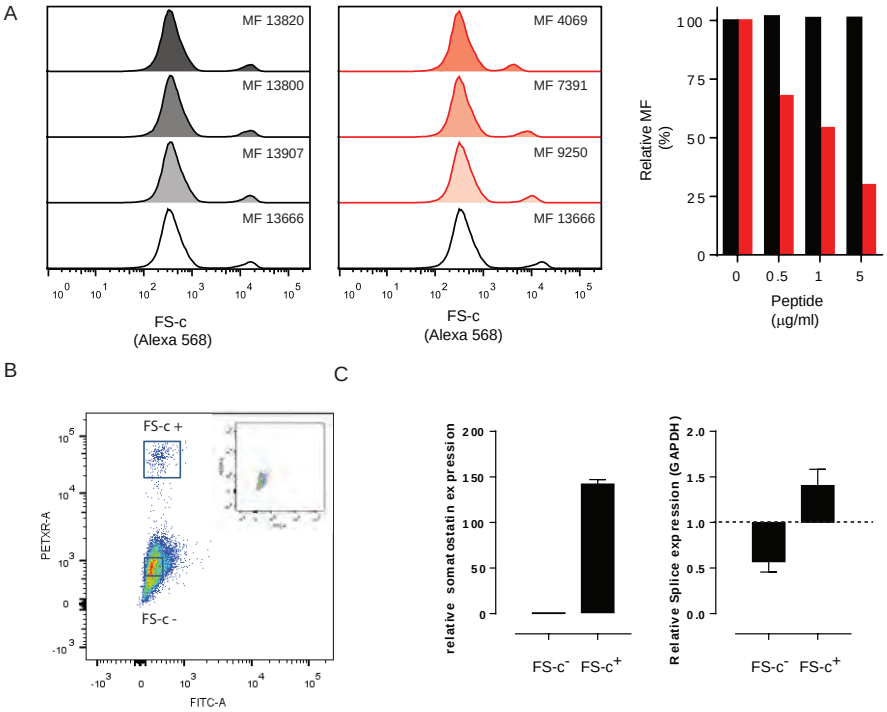

Figure 5: In depth analysis of human pancreatic FS-c$^+$ cells. A) Flow cytometric analysis of human pancreatic islets stained with FS-c serum after antibody blocking with an increasing amount of irrelevant peptide (PPI15-24) (black histograms) and the peptide used for immunization (red histograms). On the top right the mean fluorescence (MF) of the FS-c$^+$ cell population is depicted. The relative MF of the FS-c$^+$ cell population compared to unblocked antiserum (0 peptide) of antibody blocked with irrelevant peptide (black) and relevant peptide (red) (left graph). B) Sorting of FS-c$^+$ human pancreatic islet cells. Inset depicts unstained cells. C) Transcriptome analysis of FS-c$^-$ and FS-c$^+$ human islet cells for relative somatostatin and relative INS-splice expression.

Expression of INS-splice in delta cell granules

Since the alternatively spliced insulin transcript-encoded isoform has the same N-terminus as insulin, we wondered to what extent the presence of the signal peptide contributes to the posttranslational processing and localization of the isoform protein. Detailed examination of pancreatic coupes by high-resolution electron microscopy with immunogold labelled anti-serum demonstrates that the polypeptide localizes within secretory granules of delta cells (Fig. 6A,B)[29]. This confirms that INS-splice is transported to the delta cell granules and suggest it to be a secreted protein.

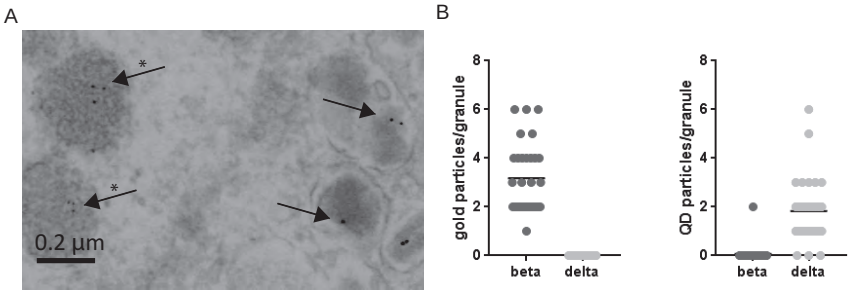

Figure 6: INS-splice is targeted to the delta cell granules. A) Electron microscopic images of human pancreatic sections labelling for INS-SPLICE (quantum dots, black * arrow) and insulin (immunogold, black arrow) visible as black dots. The granules were identified by the morphology of the secretory granules. B) Quantification of the gold$^+$ and QD$^+$ granules in beta and delta cells. Each granule is represented as a point. The graphs represent the mean of 30 beta and 30 delta cell granules.

DISCUSSION

Insulin production and secretion by beta cells is crucial for the maintenance of glucose homeostasis. Paradoxically, this hormone is also a major target in the CD4$^+$ and CD8$^+$ T cell-mediated destruction of beta cells in type 1 diabetic patients [30]. While insulin is widely studied for its role in glucose homeostasis and islet autoimmunity in T1D, little is known about other insulin gene-derived products or splicing isoforms. In this study, we investigate two insulin gene-derived polypeptides; the INS-DRiP, a defective ribosomal product, and INS-splice, a protein encoded by an alternatively spliced insulin gene-derived transcript that is partly identical to the PPI and INS-DRiP proteins.

We have previously shown that INS-DRiP may participate to T1D pathology as target of CD8$^+$ T cells[28]. Supporting our previous data, the N-terminus of DRiP, containing the CD8$^+$ T cell epitope that is lacking in INS-splice, can be detected in pancreatic beta cells by immunohistochemistry. The absence of the C-terminus may imply that this protein is rapidly targeted for degradation during translation, which is in line with the classic degradation process for proteins that lack an in frame stop codon[31].

We also report that alternative splicing of insulin pre-mRNA results in a translational product that has an N-terminal region identical to PPI and a C-terminal portion that is identical to that of the INS-DRiP protein. Similar to wild type insulin mRNA, the alternatively spliced mRNA can be found abundantly in both beta and delta cells by single-cell transcriptome analyses of human pancreatic islets (available online)[32] while hormone production

appears to be cell-type exclusive. This could point to the participation of a cell type-specific translation machinery in adult endocrine cells. Yet the relevance of the insulin gene-derived mRNA and possible translates thereof in endocrine cells other than beta cells remains unclear. Several studies suggest that delta cells have an important role in beta cell development during organogenesis[33, 34]. Also, human pancreatic beta cells have been shown to change cell identity via dedifferentiation and transdifferentiation, presumably as a manner to evade harsh environmental conditions that could result in their death[11]. Alternative splicing is involved in maintaining lineage differentiation as well as maintenance of cell pluripotency and is influenced by the microenvironment [20, 35]. Whether insulin promoter activity in endocrine cells other than beta cells is a phenotypic remnant of their common progenitor cell or contributes to maintaining their endocrine cell plasticity in adolescence remains to be elucidated.

Both the INS-DRiP and INS-splice open reading frames lacks a STOP codon. Nevertheless, the INS-splice product seems to be fully conventionally synthesized and processed as it was detectable in delta cell granules specifically. The presence of the signal peptide may target the co-translational translocation of the polypeptide to the ER lumen and subsequently secretory granules [36]. Nonstop proteins targeted to the endoplasmic reticulum and mitochondria can block translocon channels and should be rapidly cleared to allow continued cellular functions. This allows the release of nonstop proteins into the organelle and to escape proteasomal degradation[37, 38]. Although other quality control and clearance mechanisms within these organelles are likely, we propose that the cotranslational translocation allows the maturation of INS-splice, explaining the detection of the C-terminal sequence in delta cells.

The observation that this insulin isoform is exclusively detected in delta cell is intriguing. Furthermore, this suggests the presence of the preproinsulin signal peptide and B-chain in delta cells, which are major targets for islet autoimmunity in T1D[39-41]. The fate of delta cells in context of type 1 diabetes is poorly investigated. While, impaired delta cell function has been reported[42], we are not aware of any reports demonstrating evidence for delta cell destruction by autoreactive CTLs. While the function of the INS-splice polypeptide remains enigmatic, its presence in secretory granules implies that this protein is secreted and could have paracrine or endocrine functions in humans.

MATERIALS AND METHODS

Cell culture

HEK293T cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Breda, The Netherlands) supplemented with 8% fetal bovine serum (FBS) (Gibco-BRL), 100 units/ml Penicillin and 100 µg/ml Streptomycin (Gibco-BRL).

DNA constructs

To generate insulin expressing vectors, PCR on human genomic DNA was isolated from HEK293T cells. The human insulin gene was cloned by PCR using the following primers: INS Fw 5'-AGCCCTCCAGGACAGGC-3' and INS Rv 5'-TTTTGCTGGTTCAAGGGCTTTATT-3'. The PCR fragment was cloned into pJet2.1 vector and subcloned into pRRL-CMV vector to generate a pLV-CMV-INS/full expressing the human INS gene. The same PCR primers were used on RNA isolated from purified human islets. The two PCR fragments obtained were cloned into pJET2.1 and subsequently cloned into pRRL-CMV-IRES GFP to generate a pLV-CMV-INS/wt-bc-GFP and pLV-CMV-INS/splice-bc-GFP. The constructs were verified by Sanger sequencing using primer located into the CMV promoter.

Polyethlenimine transfection

HEK293T cells were transfected in suspension using polyethylenimine (PEI). A transfection mixture for a 6-well consisted of 2 µg plasmid DNA and 6 µg PEI (pH 7.4) supplemented with Opti-MEM I reduced serum medium (Gibco) to a final volume of 200 µl. Mixtures were thoroughly mixed and pre-incubated for 10 min at room temperature before adding to the cell suspension, 48 hours post transfection cells were harvested for further analysis.

Reverse transcription PCR and real time analysis

RNA was isolated with TRIzol reagent (15596018, Invitrogen). To ensure RNA quality all samples were DNase treated (Invitrogen). Reverse transcription was performed using 500 ng RNA using Superscript RT III kit (Invitrogen, Karlsruhe, Germany). Gene expression was determined using the following primers: Exon 2: Fw 5'-GGCTTCTTCTACACACCCAAG -3', Rv 5'-CGGGTCTTGGGTGTGTAGAAG-3';
Splice:

Fw 5′-GGCTTCTTCTACACACCCAAG-3′,
Rv 5′-GCAGTAGTTCTCCAGCCTGCAGGTCC-3′;
Somatostatin: Fw 5′-CGTCAGTTTCTGCAGAAGTCCCTGGCT-3′,
Rv 5′-CCATAGCCGGGTTTGAGTTAGCAGATC-3′ and GAPDH;
Fw 5′-ACAGTCAGCCGCATCTTCTT-3′, Rv 5′-AATGAAGGGGTCATTGATGG-3′.
Quantitative PCR analysis was performed using the SybrGreen master mix kit (Applied Biosystems, Foster City, CA) in triplicate on an Applied Biosystems Step One Plus machine.

Generation of custom polyclonal antisera

Custom polyclonal antisera (Eurogentec, secure.eurogentec.com/speedy, Belgium) were generated by immunization of rabbits using synthetic peptides, MLYQHLLPLPAGEC (DRiP1-13, cysteine serves as anchor residue for the carrier) and LLHRERWNKALEPAK (FS-c). During the speedy immunisation programme of 28 days the animals were repeatedly injected with synthetic peptide and bled prior immunization and post immunization. Immune responses of the bleeds were tested for antigen reactivity by ELISA performed by the manufacturer.

Recombinant polypeptides

Human recombinant polypeptides were synthesized as previously described[43]. Protein encoding cDNA was obtained from human pancreatic islets by PCR and cloned in pDest17 for protein production in *Escherichia Coli* using gateway cloning technology (Invitrogen, Carlsbad, CA, USA). Recombinant proteins were purified by the His6 affinity purification tag and freeze dried. Purified polypeptides were dissolved in 0.05% acetic acid in MQ/PBS to a stock concentration of 1 mg/ml.

Enzyme-linked immunosorbent assay

Nunc maxisorp 96 well plates (Sigma-Aldrich) were coated with 1 µg/well of the desired recombinant polypeptide diluted in PBS to reach a final volume of 100 µl/well and incubated overnight at 4C. After coating, the wells were blocked with 200 µl/well 2% BSA in PBS for 1 h at room temperature (RT), followed by 2 h incubation with the primary antibody (i.e. DRiP1-13 (1:1000), FS-c (1:1000) or ms-α-C-peptide (1:2500)) of interest and subsequently 2 h with HRP-conjugated secondary antibody (i.e. anti-rabbit and anti-mouse (Santa Cruz)) for visualization. Antibodies were diluted in 1% BSA in PBS to reach a final

volume of 100 µl/well. For detection, 100 µl/well of freshly prepared substrate, 0.4 mg/ml O-phenylenediamine (P9029, Sigma) in 0.05M phosphate-citrate buffer pH 5.0, was used. After 15 minutes incubation the absorbance at 450 nm was measured. After each step, wells were aspirated and extensive washed using 200 µl/well Wash buffer (0.05% Tween-20 in PBS). This was repeated for a total of 5 washes with 1 minute soaking to increase the effectiveness of the washes.

Human islet isolation

Pancreatic islets were obtained from non-diabetic human cadaveric donor pancreata, given the islets could not be used for transplantation and research consent was present. The isolations were performed in the GMP-facility of LUMC according to the previously described protocol[44]. For experimental use, human islets were maintained in ultra-low attachment plates (Corning, NY 14831) in low glucose DMEM supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin (Gibco-BRL).

Immunohistochemistry and confocal microscopy

Paraffin embedded tissues were cut into 4 µm sections. Tissues were deparaffinized in xylene and rehydrated in a series of ethanol decreasing in concentration. Prior antibody incubation antigen retrieval was performed by autoclavation in citric buffer (pH 6.0). Sections were blocked for 1 hour with 2% normal donkey serum in PBS. Primary and secondary antibodies diluted in PBS/1%BSA were incubated subsequently for 1 hour at room temperature with additional washing steps in between. Primary antibodies against insulin (1:100), C-peptide (1:500, CBL94 Millipore), glucagon (1:200 ab10988 Abcam), somatostatin (1:500, 13-2366 Emelca Bioscience), DRiP1-13 (1:500) and FS-c (1:500). The appropriate Alexa-conjugated antibodies were used (anti-mouse, anti-rabbit, and anti-sheep). Nuclei were stained with DAPI vectashield (Vector laboratories #H-1500). Immunofluorescence was detected with a Leica SP8 confocal microscope.

Flow cytometric analysis of human pancreatic islet cells

Prior flow cytometric analysis of human pancreatic islets, a single cell suspension was prepared by dispersion using trypsin and filtration. Thereafter, the islets cells were fixed and permeabilized with 4% PFA, 0.1% saponin in PBS for 30 min

at 4 degrees. Islet cells were subsequently stained with anti-FS-c as primary antibody (1:500) and Alexa-568 conjugated secondary antibodies against rabbit (Life technologies 1:500). Antibodies were diluted in 0.1% saponin, 1% BSA in PBS and incubation time of 1 hour cold. Islet cell populations were analysed and sorted using a FACS Aria II (BD biosciences).

To isolate RNA from sorted cells, fixation, permeabilization and staining were performed in presence of RNase inhibitor following the MARIS method described by Hrvatin et al[45].

Western blot Sample preparation and immunoblotting

Cell lysates were made using Tropix lysis mix (Applied Biosystems) and the protein content was determined by Bradford reagent assay (Biorad, Veenendaal, The Netherlands). For analysis 50 µg protein sample was used. Samples were boiled with sample buffer (10% glycerol, 2% SDS, 50 mM Tris-HCl pH 6.8, 0.1% Bromophenol blue and 1% β-mercaptoethanol) for 5 minutes before loading the samples onto a 15% SDS-polyacrylamide gel. The proteins were subsequently transferred to 0.2 µm pore size Immobilon-P (Merk Millipore) and visualized by standard protocols with anti-insulin (1:1000, H-86 sc-9168), anti-C-peptide (1:1000, CBL94 Millipore) ,anti-actin (1:5000, C4 MAB1501 Merck Millipore), anti-GFP (1:2000, A11122 Invitrogen), anti-FS-c (1:1000), anti-somatostatin (1:1000, 13-2366 Emelca Bioscience).

For antibody blocking assays, the primary antibody was blocked with 5 µg recombinant polypeptide overnight at 4°C prior addition to the membranes. After this standard western blot protocols were followed.

Donor samples for electron microscopy (EM)

Electron microscopy datasets were created from nPOD donors. Additional donor details can be obtained through the JDRF nPOD online pathology database. Tissues were recovered following informed research consent from next of kin in the United States and shipped to the nPOD program at the University of Florida for processing as previously described[46-48]. All experiments were conducted under the approval of the University of Florida Institutional Review Board and the current study fulfills all requirements for tests as approved by the medical ethical review board of the University Medical Center Groningen.

Pancreas sample electron microscopy processing

Pancreas samples were fixed in cold, freshly prepared 2% paraformaldehyde-1% glutaraldehyde for 48 hours followed by transfer to phosphate-buffered saline for storage at 4°C before shipment to the Netherlands[48]. Tissue vibratome sections (~50 µm; Microm HM 650V) were post-fixed in osmium tetroxide/ potassium ferrocyanide, followed by dehydration and flat-embedding as previously reported[49]. Next, regions with islets were selected from toluidine stained 1 µm sections using light microscopy. Subsequent ultrathin (80 nm) sections were cut (UC7 ultramicrotome, Leica Microsystems, Vienna, Austria) and placed on formvar coated copper grids (Electron Microscopy Sciences, Hatfield, Pennsylvania). Finally, sections were contrasted with uranyl acetate as previously described[49, 50].

Immuno-EM

Post-embedding immunolabeling on epon with gold or quantum dots was carried out as described before[51], using rabbit-α-FSc serum.

EM acquisition and image processing (nanotomy)

Data were acquired on a Supra 55 scanning EM (SEM; Zeiss, Oberkochen, Germany) using a scanning transmission EM (STEM) detector at 28kV with 2.5 nm pixel size with an external scan generator ATLAS 5 (Fibics, Ottawa, Canada) as previously described[50, 52]. After tile stitching, data were exported as an html file and uploaded to www.nanotomy.org.

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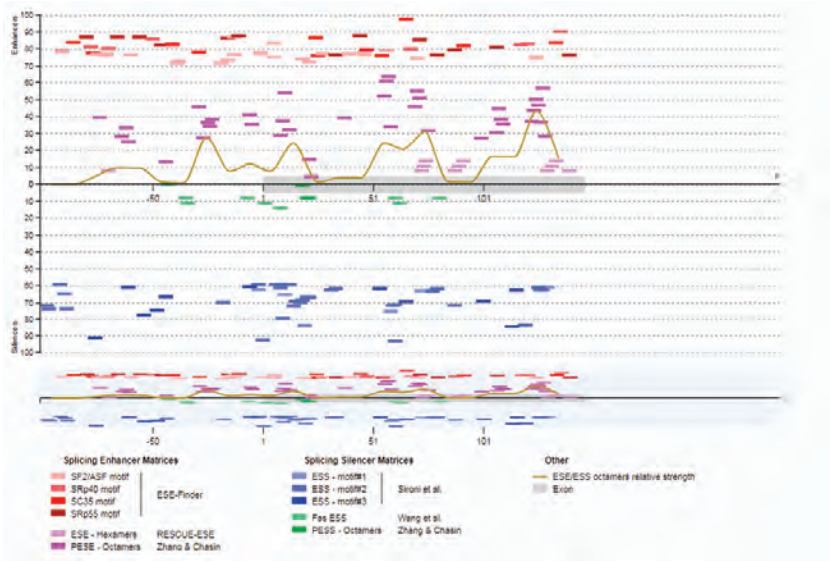
SUPPLEMENTARY INFORMATION

A



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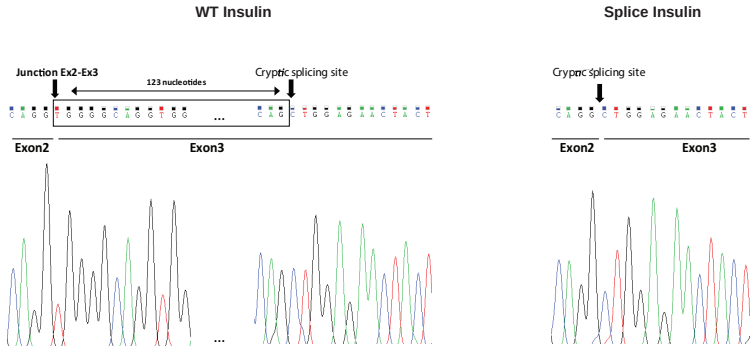

Supplementary figure 1: Alternative splice site analysis of the human insulin gene. A) Splicing analysis of the human insulin exon 3 with Human Splice Finder and ESE finder enhancers. The exon is schematically indicated by the grey bar, the intronic regions flanking the exon in a solid black line. Splicing enhancer and silence motifs are indicated by different colour bars along the sequence and further explained in the legend. The yellow line indicates the ESE/ESS relative strength. B) DNA sequence analysis of the retrieved PCR fragment spanning the splicing between 5′ donor site in exon 2 and the cryptic 3′ acceptor site in exon 3.

A

Recombinant PPI

```
  1 MSYYHHHHHH LESTSLYKKA GFTMALWMRL LPLLALLALW GPDPAAAFVN QHLCGSHLVE  ALYLVCGERG FFYTPKTRRE
 81 AEDLQVGQVE LGGGPGAGSL QPLALEGSLQ KRGIVEQCCT SICSLYQLEN YCN*
```

Recombinant INS-DRiP

```
  1 MSYYHHHHHH LESTSLYKKA GFTMLYQHLL PLPAGELLQL DAARRQPPTR RLLHRERWNK ALEPAK*
                                   DRiP1-13                 FS-c
```

Recombinant INS-splice

```
  1 MSYYHHHHHH LESTSLYKKA GFTMALWMRL LPLLALLALW GPDPAAAFVN QHLCGSHLVE ALYLVCGERG FFYTPKTRRE
 81 AEDLQAGELL QLDAARRQPP TRRLLHRERW NKALEPAK*
                                   FS-c
```

Recombinant proSST

```
  1 MSYYHHHHHH LESTSLYKKA GFTMAPSDPR LRQFLQKSLA AAAGKQELAK YFLAELLSEP NQTENDALEP EDLSQAAEQD
 81 EMRLELQRSA NSNPAMAPRE RKAGCKNFFW KTFTSC*
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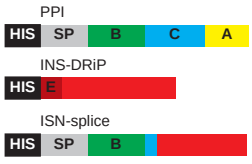
B



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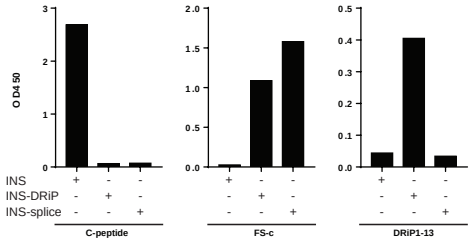
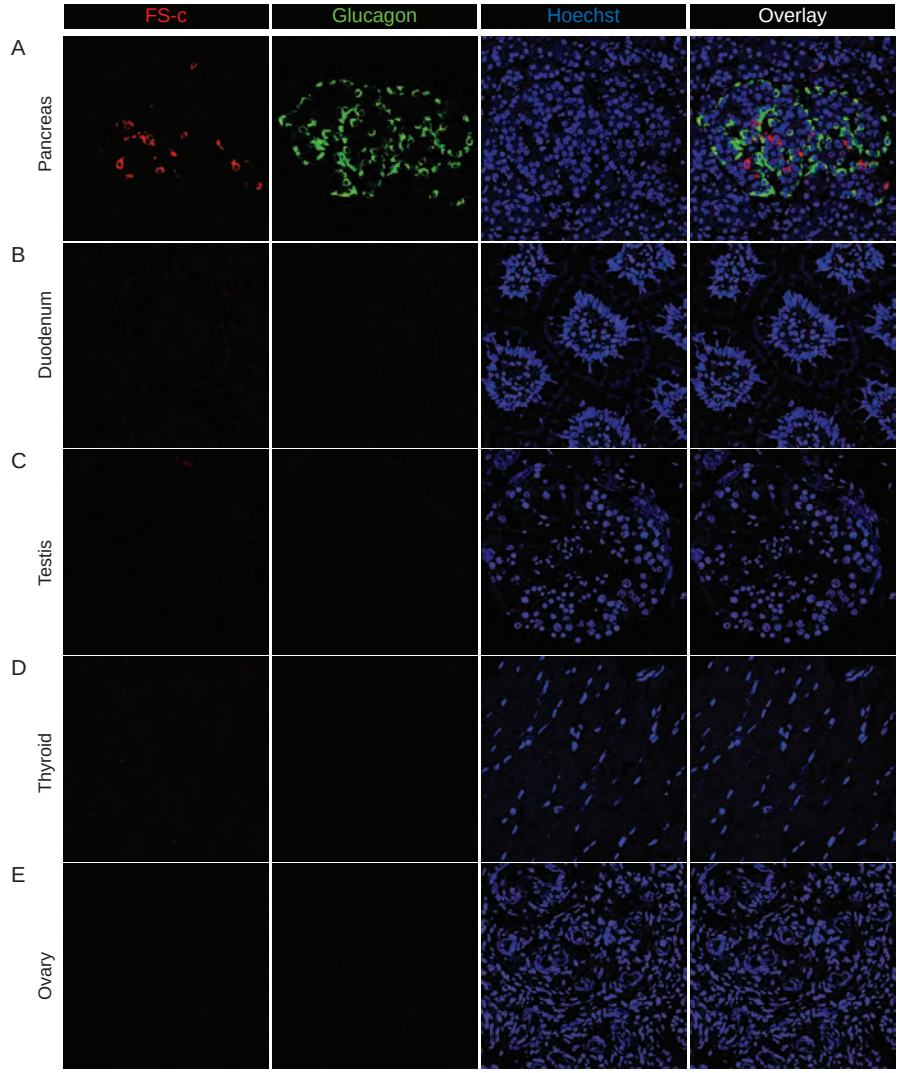
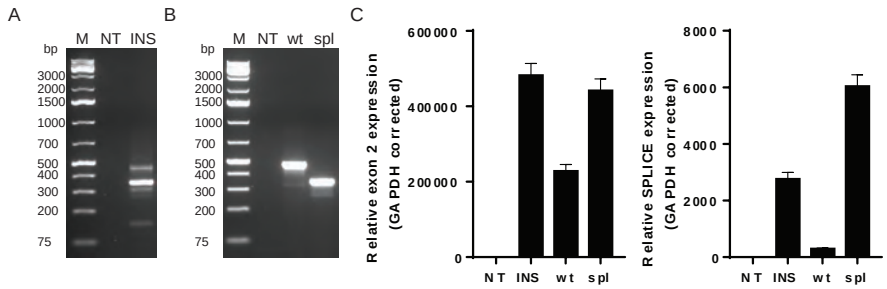


Supplementary figure 2: Recombinant polypeptides. A) Amino acid sequence of the recombinant polypeptides. Red underlined the location of the immunization peptides used for antiserum development are indicated. B) Schematic representation of the recombinant polypeptides. The His tag (black box), signal peptide (SP, grey), B-chain (B, green), C-peptide (C, blue), A-chain (A, Yellow) frame shift of INS-DRiP and INS-splice (red) are separately indicated. Corresponding amino acid sequences are indicated with corresponding colours, letters indicate the presence of the complete chain. The E indicates the INS-DRiP-specific CD8 T-cell epitope. C) Validation of serum specificity in an indirect ELISA assay to recombinant PPI, INS-DRiP and INS-splice using c-peptide antibody (left panel), FS-c antiserum (middle panel) and DRiP1-13 antiserum (right panel).



Supplementary figure 3: INS-splice is exclusively expressed in pancreatic tissue. Immunohistochemistry analysis of a variety of endocrine tissue for ins-splice (red) and glucagon (green). A) pancreas, B) Duodenum, C) Testis, D) Thyroid and E) Ovary. Nuclei are visualized by hoechst staining (blue).



Supplementary figure 4: Insulin splicing in transfected 293T cells. A) Analysis of insulin gene-derived transcript splicing in 293T cells after transfection with CMV-INS/full (INS) by PCR using the same primer set as described in Fig 1. B) Splicing analysis of transfected 293T cells with CMV-INS/wt-bc-GFP (wt) and CMV-INS/splice-bc-GFP (spl). Splicing patterns are compared to non-transfected 293T cells (NT). The DNA marker is indicated by the M. C) Primer specificity tested on RNA isolated from transfected 293T cells with CMV-INS/full (INS), CMV-INS/wt-bc-GFP (wt) and CMV-INS/splice-bc-GFP (spl) for exon 2 (right graph) and splice (left graph). All data are expressed as relative expression to non-transfected cells (NT) and GAPDH corrected.

Chapter 7

General discussion

Type 1 diabetes (T1D) results from the immune-mediated destruction of the insulin-producing beta cells. Beta cell stress and dysfunction before disease manifestation, repositions the beta cell to the center of T1D pathology and suggests the immune attack on beta cells is a well-intended response to protect the host for dysfunctional cells. The islet microenvironment leading to endoplasmic reticulum (ER) stress is a crucial component in regulating autoantigen processing (chapter 2) and neoantigen generation (chapters 3 & 4). Identification of the molecular mechanism underlying beta cell dysfunction is critical to design novel therapeutics to restore beta cell ER homeostasis, improve function and thereby reduce beta cell visibility to the immune system. The specific beta cell stress reporter assay provides a screening platform that will aid in the evaluation of compounds relieving ER stress (chapter 5). The expression of an insulin-derived isoform in delta cells (chapter 6) could present new hope for regenerative medicine and cell replacement therapy while also implying the participation of delta cells in the development of autoimmunity.

ISLETS UNDER ATTACK: WHY BETA CELL-SPECIFIC DESTRUCTION?

The unique cytoarchitecture of human islet of Langerhans contributes to the harmonized endocrine cell activities which are they key to glycaemic homeostasis[1]. The specific destruction of beta cells in T1D directed research to revolve around beta cell autoimmunity, apparently ignoring the other endocrine cell types within the Islet of Langerhans. We propose a model in which the inflammatory microenvironment combined with the physiological stress imposed by the beta cell function drives the immune attack. However, it is curious that the other endocrine cells in the islet of Langerhans that reside in the same microenvironment and have similar hormone secreting obligations seem unaffected by the immune system. Yet, several studies report that post T1D onset, glucagon-secreting alpha cells and somatostatin-secreting delta cells are impaired in their functions[2-5]. This is most likely caused by the disruption of intracellular connections and paracrine regulation within the islet in the absence of beta cell function. Intriguingly, beta cells appeared to be more sensitive to metabolically, immunologically and chemically challenging conditions than alpha cells, indicating that alpha cells are better equipped to

deal with ER stress (figure 1)[6, 7]. This might explain why the beta cell is singled out by the immune system.

Mature endocrine cells, all derived from common progenitor cells, are established early in life and barely proliferate throughout life[8]. Remarkably, the mature pancreatic endocrine cells maintain a certain degree of plasticity. After metabolic and mechanical stress spontaneous dedifferentiation and transdifferentiation of human beta cells was observed[9-11]. This endocrine plasticity is thought to allow dysfunctional beta cells to escape apoptosis (beta to alpha/delta cell conversion) due to environmental stress as well as compensate for the lost beta cell mass after destruction (alpha/delta cell to beta cell conversion)[12, 13]. The molecular processes that drive differentiation is unclear, although it appears that a switch in transcription factors expression[14] and activation of the pluripotency-related microRNA cluster are involved[15]. In addition, several studies indicate that many endocrine cells have multiple distinct hormonal transcripts, although hormone-production is largely limited to a single hormone type per cell[16-18]. In this thesis, we demonstrate the presence of a protein encoding insulin mRNA isoform in human pancreatic islets. This isoform mRNA is detected in beta cells but also in delta cells, while protein is detected only in the delta cells. This raises several questions regarding the origin, function, and consequence of insulin and insulin variants in alpha and delta cells. Is this simply a remnant of endocrine cell development? Could this contribute to endocrine cell plasticity? Does the production of an insulin splice variant in delta cells make delta cells also target for immunity? Is the insulin splice variant secreted and, if so, what receptor does it bind to and what is the effect? The answers to these questions could aid in our understanding of endocrine cell development, beta cell dysfunction, and potentially direct *in vitro* beta cell regeneration protocols.

NEOEPITOPES: DRIVERS OF DISEASE PATHOGENESIS?

The interest in neoepitopes and their role in T1D pathology is rapidly increasing. The generation of this class of epitopes is highly influenced by the environment of peripheral tissues, and this may explain why circulating T-cell have not acquired tolerance to these peptides. Therefore, neoepitopes might form the missing link between disease susceptibility, beta cell dysfunction, loss of peripheral tolerance, and disease progression[19-21].

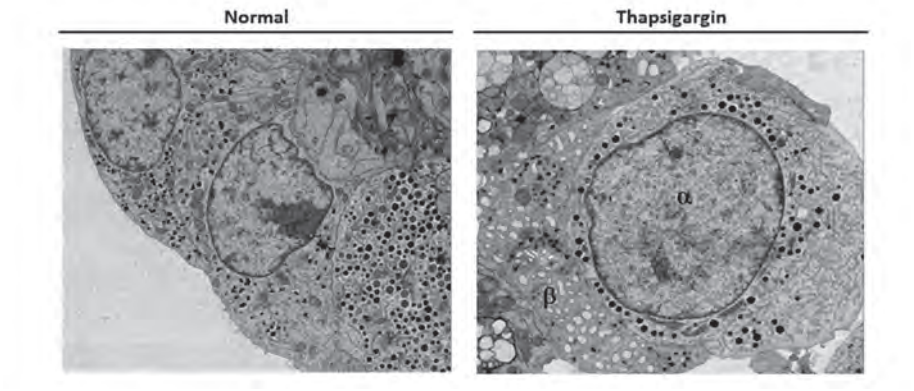


Figure 1: Human beta cells are less resistant to ER stress compared to alpha cells. Representative electron microscopy images of human pancreatic islets in normal culture conditions (left panel) compared to chemical induced ER stress by 1 μM thapsigargin for 5 hours (right panel). Alpha and beta cells are identified based on the phenotype of their granules. In thapsigargin treated samples, the swollen ER of beta cells clearly demonstrates ER stress, while this is not present in alpha cells.

Initially, the discovery of neoepitopes focussed on posttranslational modification (PTM) of native beta cell proteins, due to altered activity of enzymatic posttranslational processes upon ER stress. However, recent discovery of neoepitopes derived from alternative translation initiation of insulin mRNA[22], transpeptidation[23, 24], and fusion of two non-contiguous peptides of the same protein (cis-splicing) or distinct proteins (trans-splicing), proves that the origin of these neoepitope can be far more complex. This raises a strategic problem: where do we start looking? A powerful tool in immunopeptidome analysis is tandem liquid chromatography-mass spectrometry (LC-MS/MS) after immunoaffinity purification of peptide-HLA class I complexes[25]. It has been determined that a large fraction of the HLA class I peptides are the result degraded defective ribosomal products (DRiPs)[26] and approximately one third of transpeptidation[27]. However, our databases for spectrum identification are based on proteome or proteogenomic references thereby ignoring aberrant translational products and non-contiguous peptides[28]. To overcome this issue, special databases of predicted splice and fusion products are necessary besides the regular proteome database. Of note, these databases are based on prediction algorithms and the used techniques and methodologies greatly impact the composition of this database[29, 30]. The first results of this approach are promising as peptidome analysis of the immortalized human HLA-A2$^+$ beta cells, ECN090, indeed yielded several non-contiguous peptides[31]. It will

be difficult to translate this technique to primary human pancreatic islets, not to mention patient-derived islets, due to their limited availability for research. Caution should be taken in translating cell line-derived observations to clinical relevance. Nonetheless, immunopeptidome analysis might provide critical knowledge on the dynamics of the peptidome and how this is affected by ER stress. Unravelling the immunopeptidome could provide more understanding in the fundamental aspects of T1D pathology, biomarkers and therapeutic strategies.

IMPLICATION FOR IMMUNOTHERAPY IN T1D

A century after the first trials with exogenous insulin administration as treatment for T1D patients, this remains the standard to date. Although our knowledge on the disease pathology has vastly evolved over the last decades, strategies to prevent or cure diabetes remain beyond our capabilities. A difficult factor in the treatment of T1D is the heterogeneity among patients in pathology, disease progression, and efficacy of therapeutic intervention[32].

1. T1D biomarkers

Patients vary in genetic susceptibility, immunological background, and beta cell fragility. Therefore disease markers are likely person- and time-dependent. To utilize this knowledge we should aim for biomarkers assays that incorporate these factors to categorize patients enabling personalised therapies. Besides the classic genetic and autoantibody biomarker assays, T-cell and beta cell markers could provide a valuable contribution[33, 34].

T-cells are predominant mediators in T1D pathogenesis. Antigen-specific circulating T-cells can be measured in the peripheral blood using recombinant islet antigens[35, 36]. Discovery of novel disease-related epitopes would expand the diabetogenic T-cell repertoire that can be implemented for screening and provides a better picture of disease stage and development per individual. Of note, circulating autoreactive T-cells, especially to neoantigens, are also part of the T-cell repertoire in healthy individuals since they are not subject to central tolerance[31]. Inclusion of phenotypical characterization could indicate whether they are pathogenic[22, 31, 37]. In addition, the growing evidence of beta cell dysfunction in the pathogenesis of T1D means monitoring beta cell

functionality could be an important indicator[38]. Beta cell ER stress has been associated with accumulation and secretion of proinsulin[39, 40]. Measurement of the proinsulin/c-peptide (PI/C) ratio could be a useful indicator for beta cell dysfunction and might be detectible prior disease onset and progression[41-43]. Another non-invasive marker for beta cell stress are microRNAs[44, 45]. MicroRNAs are short, noncoding RNA molecules that have important roles in regulating almost all cellular processes, including insulin synthesis, insulin secretion, proliferation, differentiation, and apoptosis. Circulating microRNAs can be detected in all types of body fluid and are very stable[46]. Although beta cell-specific miRNAs have not yet been identified, microRNA profiles in sera of recent onset T1D patients differed from healthy donors, supporting their potential use as predictive biomarkers [47, 48]. Although the field of T-cell and beta cell biomarkers is only in its infancy, we suspect that markers for T cell and beta cell (dys)function could be valuable in early diagnosis, definition of disease heterogeneity, and stratifying patients for therapeutically strategies.

2. Re-establishing peripheral tolerance

The critical role of the immune system in beta cell destruction made immunomodulatory therapies an attractive approach. Clinical trials with immunosuppressive drugs demonstrated transient efficacy with elongated beta cell preservation in the treated group. However, long-term treatment led to adverse off-target effects and nephrotoxicity[49]. Antigen-specific immunomodulation could be a safer approach, in which unwanted T-cell responses are specifically inhibited while maintaining immunocompetence to pathogens[50]. Mapping the diabetogenic immunopeptidome is essential to establish well-defined and clinically relevant targets[51]. Antigen-specific immunotherapy includes generation of antigen-specific tolerogenic dendritic cells (tolDCs)[52, 53] or antigen-specific regulatory T cells (T_{regs})[54]. T_{regs} are crucial in maintaining self-tolerance and preventing autoimmunity via suppression of self-reactive T-cells. Tolerogenic DCs can be generated *in vitro* from PBMC-derived precursors[55] and antigen-specific T_{regs} can be established from pluripotent stem cells *ex vivo*, providing a renewable source for treatment[56-58]. In case of patient-derived resources it should be considered that dysfunction of these cells might cause reduced immunosuppression[59, 60]. Furthermore, the dynamic phenotype of these immune cells might make it difficult to use this as a long-lasting therapy.

3. Restore beta cell function and ER homeostasis

The emerging role of beta cell dysfunction prior to clinical signs of type 1 diabetes urges to make us reconsider our therapeutic strategies. If beta cells dysfunction drives the break in peripheral tolerance, this would imply that restoring beta cell function prevents diabetes development. Recently, it was demonstrated that direct inhibition of inositol-requiring enzyme 1α (IRE 1α), an ER stress sensor and activator of the unfolded protein response (UPR), reduces ER stress and reverses autoimmune diabetes in mice[61]. Of course, it is unethical to treat individuals at risk without clinical symptoms of disease. Nonetheless, several studies indicate that T1D patients have remaining beta cell mass with persistent low-level insulin or proinsulin secretion even in long-standing T1D patients [62-65]. These beta cells are most likely in a dormant state and thereby evade the immune attack. Reactivating these beta cells might restore glucose homeostasis and cure diabetes in these individuals. In this case, we should consider to combine immune therapy with beta cell therapy, to prevent a relapse. In long standing T1D patients or patients with insufficient beta cell mass, a similar approach can be envisaged in combination with beta cell replacement, such as islet cell transplantation.

CONCLUSION

The pathophysiology of T1D is extremely complex. Genetic predisposition, impaired immune regulation, and beta cell (dys)function all contribute to disease initiation and progression. A critical gap in our knowledge is what causes the break in peripheral tolerance that eventually leads to beta cell destruction. We propose that neoepitopes generated by dysfunctional beta cells activate immune surveillance, causing full-blown beta cell autoimmunity. ER stress imposed both by intrinsic beta cell physiology and by external secondary triggers seems to be a crucial component in this process. Understanding the molecular mechanisms underlying beta cell dysfunction and neoantigen generation is critical to identify clinically relevant neoepitopes. This subsequently provides more insight in the disease dynamics as well as contribute to translational research in the development of biomarker assays and development of therapeutic strategies targeting autoreactive T-cells and beta cell function. Our task will be to restore the balance between immune

reactivity and beta cell function, in order to prevent, treat, or cure type 1 diabetes (figure 2).

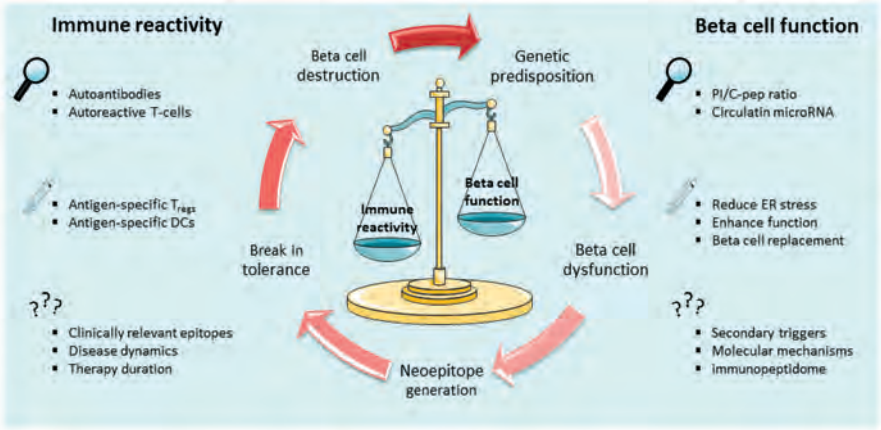


Figure 2: The viscous cycle of beta cell destruction. Beta cell dysfunction might be the driving force in disease onset in predisposed individuals, mainly by the generation of neoepitopes ultimately leading to a break in tolerance, amplification of autoimmunity and beta cell destruction. Figure was produced using Servier Medical Art www.servier.com.

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CHAPTER 8

Scientific summary

Type 1 Diabetes (T1D) is the consequence of the progressive destruction of the pancreatic beta cells by autoreactive $CD8^+$ T cells, resulting in insulin deficiency and impaired blood glucose homeostasis. Risk of developing T1D has been associated with genetic factors, however genetic susceptibility is not sufficient to drive disease pathology. Endoplasmic reticulum (ER) stress in dysfunctional beta cells caused by environmental triggers is likely involved in breaking peripheral tolerance and drive disease pathogenesis. The ER is essential for insulin production and secretion by beta cells. Perturbations of the ER and the accumulation of unfolded proteins, lead to activation of the unfolded protein response. Signaling cascades induced by the activated ER sensors PERK, ATF6, and IRE1α attempt to reduce the ER load and increase the ER capacity in order to restore homeostasis. In this thesis, the effect of the inflammatory microenvironment on human beta cell function and immunogenicity has been investigated.

Insulin is believed to be a primary autoantigen involved in beta cell destruction in T1D patients. The signal peptide of preproinsulin (PPI) is a major target of $CD8^+$ T cells. Generation and presentation of this epitope, $SP_{15\text{-}24}$, is mediated via signal peptide peptidase and ER aminopeptidase 1 (ERAP1). In **chapter 2**, we demonstrated that ER stress induced by proinflammatory cytokines facilitates the processing of $SP_{15\text{-}24}$, via upregulation of ERAP1. This upregulation was mediated by a decrease in miR-17 via IRE1α RNase activity. MiR-17 overexpression or IRE1α inhibition blunted the effect induced by proinflammatory cytokines. These data provide direct evidence of the implication of ER stress in autoantigen processing and beta cell immunogenicity.

T1D was previously assumed to be caused by impaired thymic T cell education. However, the implication of beta cell dysfunction prior to disease onset rather supports an alternative explanation where the beta cells actively contribute to their demise. Besides invading pathogens, the immune system is also trained to eliminate dysfunctional cells to prevent them from becoming harmful for the entire organism. A well-known example of this is an effective antitumor response. In **chapter 3.1** we compared the tumor microenvironment with the microenvironment of islets during insulitis and argue that diabetes might be result of successful clearance of dysfunctional cells by the immune system. Neoepitopes are important T cell targets in an effective anti-tumor response as they discriminate functional-self from dysfunctional-self. Cancerous cells have been shown to generate a wide variety of neoepitopes resulting from

mutations, splice variants, defective ribosomal products, and posttranslational modification (PTM) of native proteins. In **chapter 3.2** the contribution of post-transcriptional and post-translational modifications in the generation of beta cell neoantigens is evaluated. Compelling evidence supports the role of environmental stress-induced neoepitope formation in T1D pathogenesis. Altogether, these examples suggest that beta cell destruction resembles an effective anti-tumor response. A better understanding of the processes and the origin of neoepitopes is essential in our battle against T1D.

Based on this hypothesis, in **chapter 4** we investigated insulin as source of neoepitopes generated by unconventional translational processes. The high translation rate of insulin combined with the intrinsic physiological production pressure during metabolic needs, made this an interesting candidate. This led to the identification of an alternative open reading frame within insulin mRNA. Production of this defective ribosomal product (DRiP) might be associated with ER stress, as was observed in surrogate beta cells. Interestingly the resulting polypeptide contains an immunodominant N-terminus that can be presented in HLA class I and class II. $CD8^+$ T cells directed to this epitope were found in a higher frequency in the circulation of patients. Furthermore, these $CD8^+$ T cells killed human pancreatic beta cells *in vitro* which was enhanced during inflammatory conditions, underscoring its relevance to disease pathogenesis.

Chapters 2, 3 and 4 emphasize the importance of ER stress in the processing and generation of beta cell (neo-)epitopes and autoimmunity. Therefore, the ability to monitor ER stress is essential in understanding disease pathogenesis and might contribute to the development of therapeutics. In **chapter 5,** we designed a bioluminescent reporter assay for monitoring ER stress in human beta cells. This reporter construct encodes a XBP1-Gaussia luciferase fusion protein. Endogenous XBP1 is unconventionally spliced by IRE1α upon ER stress, resulting in the shift of the reading frame and therefore translation of an elongated C-terminal protein. Similarly, in this fusion construct, the C-terminal Gaussia Luciferase is only in the correct reading frame after IRE1α-mediated splicing, resulting in stress-induced Luciferase expression. ER stress quantification was accurate when compared to classical ER stress quantification methods and less laborious in execution. In addition, reporter expression driven by the human insulin promotor restricts the reporter to beta cells specifically. Therefore this assay allows specific monitoring of the ER stress status of beta cells in isolated human pancreatic islets. We envisage that this reporter can be

used in studies on the origin of ER stress, as well as a drug screening platform to reduce ER stress.

Insulin-production by the beta cells is of vital importance, paradoxically, as stated earlier, insulin remains also a main autoantigen in T1D. Besides insulin and INS-DRiP, we discovered an alternative insulin gene-derived splice variant (INS-splice) that contains the PPI signal peptide, the B-chain and the C-terminus of INS-DRiP. In **chapter 6,** we analysed the expression of these insulin variants in human pancreatic islets using specific custom-made antibodies. In accordance with our previous results, the N-terminus of INS-DRiP containing the $CD8^+$ T cell epitope, was detected in pancreatic beta cells. Yet, the C-terminus of INS-DRiP was not detected, implying that the polypeptide does not exist as mature polypeptide, maybe as consequence of rapid nonSTOP protein decay. The mRNA encoding INS-splice is present in beta and delta cells, however, the polypeptide appears restricted to delta cells. More specifically, the INS-splice polypeptide was localized in the delta cell granules. We suspect this might be the consequence of the presence of the PPI signal peptide in INS-splice, which drives the co- and posttranslational processing. Although the function of INS-splice remains elusive, we envision it might take part in beta and delta cell lineage development, while it is conceivable that it contributes to maintaining mature endocrine cell plasticity as alternative splicing is an important mechanism in lineage development and maintaining pluripotency. Its localization in delta cell granules also suggest a potential paracrine or endocrine function.

ER stress in beta cells seems to drive T1D etiology by attracting immune cells, increased autoantigen processing, and neoepitope generation. Neoepitope formation might have a crucial role in the loss of peripheral tolerance and beta cell destruction. Therefore identification of these neoepitopes is important for monitoring disease progression, as well as for the development of immunotherapies in order to re-establish peripheral beta cell tolerance. In addition, our results point to the importance of research to the causes of beta cell dysfunction and propose restoration ER homeostasis as potential therapeutic target.

ADDENDUM

Nederlandse samenvatting
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Dankwoord

NEDERLANDSE SAMENVATTING

Type 1 Diabetes (T1D) is het gevolg van de progressieve vernietiging van de bètacellen in de alvleesklier door autoreactieve $CD8^+$ T-cellen. Dit leidt tot een tekort aan insuline en verstoorde homeostase van bloedglucose. Erfelijke factoren kunnen een verhoogd risico geven op het ontwikkelen van T1D, maar de genetische aanleg is niet voldoende om de ziekte te veroorzaken. Endoplasmatisch reticulum (ER) stress in disfunctionele bètacellen veroorzaakt door omgevingsprikkels is waarschijnlijk betrokken bij het doorbreken van perifere tolerantie en het initiëren van de pathogenese. Het ER is essentieel voor insuline productie en uitscheiding door bètacellen. Verstoringen van het ER en de accumulatie van ongevouwen eiwitten leiden tot activering van de ongevouwen eiwit respons, door activatie van ER-sensoren PERK, ATF6 en IRE1α. Deze sensoren induceren signaleringsketens die trachten de ER-belasting te verminderen en de ER-capaciteit te vergroten om de homeostase te herstellen. In dit proefschrift is het onderzoek naar de effect van het inflammatoire micromilieu op de humane bètacelfunctie en immunogeniteit beschreven.

Insuline wordt beschouwd als een belangrijk autoantigeen dat betrokken is bij de vernietiging van bètacellen bij T1D-patiënten. Het signaalpeptide van preproinsuline (PPI) is een belangrijk doelwit van $CD8^+$ T-cellen. Vorming en presentatie van het epitoop $SP_{15\text{-}24}$ wordt mede tot stand gebracht via signaalpeptidepeptidase en ER-aminopeptidase 1 (ERAP1). In **hoofdstuk 2** hebben we aangetoond dat ER stress geïnduceerd door pro-inflammatoire cytokinen de verwerking van $SP_{15\text{-}24}$ vergemakkelijkt via opregulatie van ERAP1. Deze opregulatie werd gemedieerd door een afname in miR-17 via IRE1α RNase-activiteit. MiR-17 overexpressie of remming van IRE1α verminderde de insult veroorzaakt door pro-inflammatoire cytokinen. Deze resultaten leveren direct bewijs voor de bijdrage van ER-stress bij de auto-antigeen verwerking en de immunogeniteit van bètacellen.

T1D werd geacht te worden veroorzaakt door onvolledige T-cel selectie in de thymus.Echter, het aandeel van bètacel disfunctie voorafgaand aan ziekte verschijnselen maakt plaats voor een alternatieve verklaring waarin de bètacellen actief bijdragen aan hun ondergang. Naast het opruimen van binnendringende ziekteverwekkers, is het immuunsysteem ook getraind om disfunctionele cellen te elimineren om zo te voorkomen dat ze schadelijk worden voor het lichaam. Een bekend voorbeeld hiervan is een effectieve

antitumor reactie. In **hoofdstuk 3.1** vergeleken we het tumor micromilieu met het micromilieu van eilandjes van Langerhans tijdens ontsteking ('insulitis') en betogen dat diabetes mogelijk het resultaat is van succesvolle verwijdering van disfunctionele cellen door het immuunsysteem. Neo-epitopen zijn belangrijke doelwitten van T-cellen in een effectieve antitumor respons omdat op deze manier 'functioneel-zelf' van 'disfunctioneel-zelf' kan worden onderscheiden. Van kankercellen is aangetoond dat ze een grote variatie aan neo-epitopen genereren door middel van onder andere mutaties, alternatieve splicing, foutieve ribosomale producten en veranderingen in posttranslationele modificatie (PTM) van natieve eiwitten. In **hoofdstuk 3.2** wordt de bijdrage van post-transcriptionele en post-translationele modificaties in de vorming van bètacel neo-antigenen geëvalueerd. Er zijn steeds meer aanwijzingen die de rol van omgevingsstress in neo-epitoop vorming in T1D pathogenese ondersteunen. Deze voorbeelden tezamen suggereren dat de vernietiging van bètacellen op een effectieve antitumor respons lijkt. Een beter begrip van de oorsprong van neoepitopen is essentieel in onze strijd tegen T1D.

Op basis van deze hypothese hebben we in **hoofdstuk 4** insuline onderzocht als bron van neo-epitopen die zijn gegenereerd door onconventionele translationele processen. De hoge translatie snelheid van insuline in bètacellen, in combinatie met de intrinsieke fysiologische productiedruk tijdens metabolische behoeften, maken insuline een interessante kandidaat. Dit leidde tot de identificatie van een alternatief open leesraam binnen het insuline-mRNA. De productie van dit defecte ribosomale product is geassocieerd met ER-stress, zoals werd waargenomen in surrogaat bètacellen. Interessant is dat het resulterende polypeptide een zeer immunodominante N-terminus bevat dat kan worden gepresenteerd in zowel HLA klasse I als klasse II. $CD8^+$ T-cellen gericht tegen dit epitoop werden in een hogere frequentie in de circulatie van patiënten gevonden. Bovendien waren deze $CD8^+$ T-cellen in staat menselijke bètacellen geïsoleerd uit de alvleesklier te vernietigen *in vitro*. Dit proces werd versterkt door ontstekingsfactoren duidend op de mogelijke relevantie hiervan in het ziekteproces.

Hoofdstukken 2, 3 en 4 benadrukken het belang van ER stress bij de verwerking en vorming van bètacel (neo)epitopen en auto-immuniteit. Daarom is het vermogen om ER stress te monitoren essentieel voor het begrijpen van het ziekteproces en kan het bijdragen aan de ontwikkeling van behandelingstherapieën. In **hoofdstuk 5** hebben we een bioluminescente

reporter-test ontworpen voor het ER-stress in menselijke bètacellen te detecteren. Dit reporterconstruct codeert voor een XBP1-Gaussia luciferase fusie-eiwit. In stress situaties wordt endogeen XBP1 geknipt door IRE1α, wat een verschuiving van het leesraam veroorzaakt waardoor gedurende translatie de C-terminus van het eiwit wordt verlengd. Op een vergelijkbare manier zit de C-terminale Gaussia luciferase in dit fusieconstruct alleen in het correcte leesraam na IRE1α gemedieerde splicing, wat resulteert in stress geïnduceerde expressie van luciferase. De kwantificatie van ER stress bleek gelijkwaardig aan klassieke ER stress-kwantificeringsmethoden, maar was minder bewerkelijk in uitvoering. Bovendien kan met behulp van een humane insuline promotor, de reporter specifiek tot expressie worden gebracht in bètacellen. Zodoende kan specifiek de ER stress status van bètacellen in geïsoleerde menselijke pancreaseilandjes worden bepaald. We voorzien dat deze reporter kan worden gebruikt om de oorsprong van ER stress te begrijpen en als screeningplatform voor het ontwikkelen van geneesmiddelen om ER stress te verminderen.

Insuline productie door bètacellen is van cruciaal belang, tegenstrijdig hieraan is dat insuline ook een van de belangrijkste autoantigenen is in T1D. Naast insuline en INS-DRiP, hebben we een splice variant van het insuline gen ontdekt dat bestaat uit de PPI signaalpeptide, de insuline B-chain en de C-terminus van INS-DRiP. In **hoofdstuk 6** analyseerden we deze insuline varianten in menselijke eilandjes van Langerhans met behulp van specifieke custom-made antisera. In overeenstemming met onze eerdere resultaten werd de N-terminus van INS-DRiP, waar het CD8 T-cel epitoop zich bevindt, gedetecteerd in bètacellen. De C-terminus van INS-DRiP werd niet gedetecteerd, wat erop kan duiden dat het polypeptide niet als volledig polypeptide bestaat, mogelijk als gevolg van snelle afbraak van 'non-STOP' eiwitten. Het mRNA dat codeert voor INS-splice is aanwezig in bèta- en deltacellen, maar het polypeptide lijkt beperkt te zijn tot deltacellen. Om specifiek te zijn, was het INS-splice eiwit gelokaliseerd in de granules van deltacellen. We vermoeden dat dit gerelateerd is aan de aanwezigheid van het signaalpeptide in INS-splice, dat de co- en posttranslationele verwerking stuurt. Hoewel de functie van INS-splice nog onbekend is, stellen wij voor dat deze splice variant mogelijk verband houdt met de ontwikkeling van beta- en deltacellen gedurende organogenese, maar het is ook denkbaar dat het bijdraagt aan het behouden van de plasticiteit van volwassen hormoon producerende cellen, aangezien alternatieve splicing een belangrijk mechanisme is bij organogenese en het

behoud van pluripotentie. De lokalisatie in deltacel granules suggereert ook een potentiële paracriene of endocriene functie.

ER stress in bètacellen lijkt T1D etiologie te stimuleren door het aantrekken en activeren van afweercellen, een verhoogde verwerking en presentatie van auto-antigenen en de productie van neo-epitopen. De formatie van neo-epitopen speelt mogelijk een cruciale rol in het verlies van perifere tolerantie. De identificatie van deze neo-epitopen is dus belangrijk voor bestudering en het begrijpen van ziekteprogressie, maar ook voor de ontwikkeling van immunotherapieën om de perifere bètacel tolerantie te herstellen. Daarnaast toont ons onderzoek het belang van onderzoek naar de oorzaken van bètacel disfunctie en stelt herstel van ER homeostase voor als potentieel therapeutisch doelwit.

CURRICULUM VITAE

Maria Jacoba Lenie (Marjolein) Kracht was born on the 10th of August 1990 in Schipluiden, the Netherlands. In 2008 she completed her pre-university education at the Christelijk Lyceum Delft, Delft, the Netherlands. The same year she started her study Life Science and Technology at Technical University, Delft, and Leiden University, Leiden, the Netherlands. Which included an internship in the laboratory of Prof. dr. Olsthoorn at the department of molecular genetics, Leiden Institute of Chemistry. After obtaining her bachelor's degree, she continued the master Life Science and Technology (Research and Development track), at Leiden University. Upon completing the master's curriculum and an internship in the laboratory of Prof. dr. Hoeben under supervision of dr. Zaldumbide at the department of Molecular Cell Biology, Leiden University Medical Center (LUMC), she obtained her master's degree *cum laude*.

In 2014 she accepted a PhD position at the department of Molecular Cell biology of Prof. dr. Hoeben and department of Immunohematology and Blood Transfusion of Prof. dr. Roep, LUMC, again under supervision of dr. Zaldumbide. The results of these studies are described in this thesis. In 2017, she was awarded Best Article Prize 2017 (non clinical) by the Executive Board of the LUMC for her report "Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes".

Marjolein will continue researching the contribution of beta cell fragility to diabetes pathogenesis in the laboratory of Translational Immunology headed by Prof. dr. Liston at the KU Leuven, Belgium.

LIST OF PUBLICATIONS

1. **Kracht MJL**, de Koning EJP, Hoeben RC, Roep BO, Zaldumbide A. Bioluminescent reporter assay for monitoring ER stress in human beta cells. *Sci. Rep. 2018; 8(1), 17738.* Doi: 10.1038/s41598-018-36142-4.

2. **Kracht MJL**, van Lummel M, Nikolic T, Joosten AM, Laban S, van der Slik AR, van Veelen PA, Carlotti F, de Koning EJ, Hoeben RC, Zaldumbide A, Roep BO. Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes. *Nat. Med. 2017; 23(4), 501-507.* Doi: 10.1038/nm.4289.

3. Roep BO, **Kracht MJL**, van Lummel M, Zaldumbide A. A roadmap of the generation of neoantigens as targets of the immune system in type 1 diabetes. *Curr. Opin. Immunol. 2016; 43, 67-73.* Doi: 10.1016/j.coi.2016.09.007.

4. **Kracht MJL**, Zaldumbide A, Roep BO. Neoantigens and microenvironment in type 1 diabetes: lessons from antitumor immunity. *Trends Endocrinol. Metab 2016; 27(6), 353-362.* Doi: 10.1016/j.tem.2016.03.013.

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