Novel players in rescue of β-cells from inflammation-mediated destruction in Type 2 Diabetes Mellitus

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Abstract

Diabetes is a metabolic syndrome characterized by failure of multiple organ systems to maintain normoglycemia. At the crux of this malfunction lies the pancreatic Islet of Langerhans, which secretes hormones responsible for regulation of circulating blood glucose levels. Not only in the autoimmune Type 1 Diabetes (T1D), but also in Type 2 Diabetes (T2D), the survival and function of the insulin secreting β-cells is diminished. In T1D, β-cell destruction occurs through immune mediated processes involving mononuclear cell infiltration in the pancreatic islets and interaction between antigen presenting cells and T-cells leading to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other inflammatory products and subsequently to β-cell apoptosis. Pro-inflammatory cytokines and chemokines are produced and secreted not only by activated infiltrating macrophages, but also by adipocytes and pancreatic β-cells themselves. On the other hand, T2D is diagnosed in individuals with relative insulin insufficiency; β-cells have lost the ability to compensate and produce sufficient insulin to maintain normoglycemia in the face of higher insulin demands. For many years, the mechanisms of β-cell apoptosis only in T1D were thought to be immune system mediated and were supposed to be distinct from the stressed β-cell death in T2D. In contrast, recent studies show local inflammation in the islets also in T2D (IL-1β, IL-6, IL-8, CXCL-10), linking T1D and T2D as inflammatory diseases.

Current therapies in diabetes lack focus on maintenance of endogenous β-cell mass. In order to come up with novel targets for improving β-cell mass, this thesis aimed to investigate the varied aspects of β-cell survival and function, with special focus on inflammation in islets.

In the first study, I revealed a novel target TOSO, which inhibited the death receptor Fas activation by restoring endogenous levels of FLIP. TOSO could not only protect the β-cells from elevated glucose mediated apoptosis, but also induced limited round of proliferation in these cells. To assess the proliferation of β-cells induced by TOSO, I adapted a novel technique called Sequential thymidine analogue labeling, to detect multiple rounds of cell division *in vitro*. Using this assay, I could confirm the low proliferative capacity of adult β-cells. Nevertheless, TOSO induced an early round of

proliferation in the β-cells. Thus, TOSO could be a potential target for protecting the β-cells and restoring their mass in the face of diabetes.

An additional aim of the thesis was to understand the effect of an existing class of drugs improving insulin secretion, the DPP4-inhibitors, on the survival and function of β-cells in islets. DPP4 is an enzyme, which cleaves the incretin GLP1, thus abolishing the potentiation of insulin secretion by the β-cells. My investigations show that the DPP4 inhibitor Linagliptin improves β-cell function and survival under diabetogenic conditions by stabilizing GLP1 and activating an anti-inflammatory/antioxidative stress pathway.

The major investigation of this thesis has been the systemic inflammation observed in diabetes and its role in β-cell apoptosis. The signaling and activation of immune cells is brought by secreted stimulators or via cell-cell interactions. Different cell surface receptors and adhesion molecules play a role in the immune system activation. One such family of adhesion and signaling molecules are Sialic acid-binding immunoglobulin-like lectins (Siglecs). Siglecs are I-type lectins. which recognize and interact with sialylated glycan residues on cell surfaces via immunoglobulin (Ig)-like domains. Siglec expression has been found mainly in the hematopoietic and immune systems. Most siglecs have one or more cytosolic immunoreceptor tyrosine-based inhibitory motif (ITIM) in the intracellular domain, suggesting a role for siglecs in signaling events through the recruitment of tyrosine and inositol phosphatases.

These cell-cell signaling interactions play an important role in immune system activation and triggering of inflammation. As a consequence, they might be initiators of the inflammatory cascade in diabetes and the deleterious interaction of the immune cells with the β-cells. Hence, exploring the role of siglecs in diabetes is necessary to understand the possible pro-inflammatory triggers of β-cell destruction. In this thesis, I investigated expression and function of siglecs in the pancreas, as well as in the immune cells, under diabetogenic conditions. Cell type specific expression of siglecs could be detected in islets in the human pancreas: Siglec-3, -5 and -8 are expressed in the glucagon producing α-cells; Siglec-1, -2, -7 and -10 are expressed in the insulin producing β-cells. Also, the β-cell specific Siglec-7 and -10 were down-regulated in diabetes, with opposite regulation of the ligands. Restoration of Siglec-7 in the β-cells could improve their function and protect them from elevated glucose, free fatty acid and

cytokine induced apoptosis by inhibiting the activation of the classical NFκB pathway and the subsequent cytokine secretion. Similar loss of Siglec-7 was observed in activated monocytes. Ultimately, the migration of immune cells towards inflamed islets could also be prevented by restoration of Siglec-7 in these islets. In summary, from our data we can predict that Siglec-7 expression is necessary for maintenance of an anti-apoptotic pro-survival state in islets, and its loss aggravates the activation of immune system and islet inflammation occurring in diabetes.

In conclusion, these investigations have brought to surface the presence of siglecs in non-inflammatory cells in pancreatic tissue, opening a whole new perspective for the role of siglecs in general. This thesis widens the understanding of islet inflammation and puts forth novel targets to protect the β-cells under these circumstances.

Zusammenfassung

Diabetes ist eine Stoffwechselerkrankung, bei der eine komplexe Fehlregulation mehrerer Organsysteme zur Erhöhung der Blutzuckerwerte führt. Ein Funktionsverlust der Langerhansschen Inselzellen des Pankreas, deren sezernierte Hormone im gesunden Organismus den Blutzucker regulieren, stellen dabei die Hauptursache der Manifestation des Diabetes dar. Sowohl im autoimmunen Typ 1 Diabetes (T1D), als auch im Übergewicht-assoziierten Typ 2 Diabetes (T2D) sind Überleben und Funktion der insulinproduzierenden β-Zellen beeinträchtigt. In T1D werden die Inselzellen von Immunzellen infiltriert; das Zusammenspiel antigenpräsentierender- und T-Zellen führt zur lokalen Entzündung mit erhöhten Konzentrationen von inflammatorischen Zytokinen, Chemokinen, reaktiven Sauerstoffspezies (ROS) und anderen Produkten und spezifischer Zerstörung der insulinproduzierenden β-Zelle in den Inseln durch programmierten Zelltod (Apoptose). Proinflammatorische Zytokine und Chemokine werden dabei nicht nur von aktivierten infiltrierenden Makrophagen sekretiert, sondern auch von β-Zellen selbst. T2D tritt in Patienten auf, die die Fähigkeit verloren haben, ausreichende Mengen an Insulin zur Aufrechterhaltung physiologischer Blutzuckerwerte in Situationen erhöhtem Insulinbedarfs (z.B. Übergewicht, Insulinresistenz) zu produzieren. Lange Zeit nahm man an, dass der Mechanismus der β-Zell-Apoptose im T1D immunsystemabhängig und damit unterschiedlich zum stressinduzierten β-Zell-Tod im T2D sei. Im Gegensatz dazu zeigen neuere Studien auch im T2D eine lokale Entzündung in den Inseln (IL-1β, IL-6, IL-8, CXCL-10), somit stellt eine Aktivierung von Entzündungsfaktoren und der daraus resultierende Tod der β-Zelle eine Gemeinsamkeit beider Hauptformen des Diabetes, T1D und T2D dar. Kausale Therapien zur Erhaltung der β-Zell-Masse stehen derzeit noch nicht zur Verfügung. Um neue Ansatzpunkte für einen Schutz der β-Zell-Masse zu erhalten, sollen in dieser Arbeit verschiedene Aspekte des β-Zell-Überlebens und –Funktion untersucht werden, mit dem speziellen Fokus auf die Hemmung der Inflammation in den Inselzellen.

In der ersten Studie untersuchte ich das Protein TOSO, ein Regulationsprotein innerhalb des pro-apoptotischen Fas Signalweges. Durch die Wiederherstellung endogener Konzentrationen von FLIP, ein Potein, das den Fas-Signalweg inhibiert, schützte TOSO die β-Zellen nicht nur vor Glukose-induzierter Apoptose, sondern erzielte außerdem eine erhöhte Proliferationrate in β-Zellen. Mittels sequentieller Thymidin-analogafärbung gelang es mir, mehrere Zyklen der Zellteilung *in vitro* zu untersuchen. Mit Hilfe dieses Assays konnte ich die niedrige Proliferationskapazität von adulten β-Zellen bestätigen. TOSO induzierte eindeutige Erhöhung der β-Zell-Proliferation, jedoch die von mir erhoffte mehrmalige Induktion der Zellteilung in spezialisierten β-Zellen konnte nicht bestätigt werden.

Ein weiteres Ziel dieser Arbeit war, den direkten Einfluss einer bereits existierenden Klasse von Medikamenten, die Inhibitoren der Dipeptidylpeptidase 4 (DPP4), auf Überleben und Funktion der β-Zelle zu untersuchen.

DPP4 ist ein Enzym, das das Inkretin GLP1 spaltet und damit die Potenzierung der Insulinsekretion in β-Zellen verhindert. DPP4 Inhibitoren erhöhen die Glukose-

induzierte Insulinsekretion. Meine Untersuchungen zeigen, dass der DPP4-Inhibitor Linagliptin das β-Zell-Überleben und deren Funktion unter diabetogenen Bedingungen verbessert, in dem er GLP1 stabilisiert und einen anti-inflammatorischen/antioxidativen Signalweg induziert.

Der Großteil der Untersuchungen dieser Arbeit konzentrierte sich auf die Entzündungsreaktionen, die bei Diabetes beobachtet werden können und dessen Rolle bei der β-Zell-Apoptose. Die Kommunikation und Aktivierung von Immunzellen wird durch sekretierte Stimulatoren oder durch Zell-Zell-Interaktionen vermittelt. In der Aktivierung des Immunsystems spielen verschiedene Oberflächenrezeptoren und Zelladhäsionsmoleküle eine Rolle. Eine Familie dieser Adhäsions- und Signalmoleküle sind die Sialic acid-binding immunoglobulin-like lectins (Siglecs). Siglecs sind I-Typ Lectine, die sialinsäurehaltige Polysaccharide auf Zelloberflächen durch immunglobolin-(Ig) ähnliche Domänen erkennen und mit ihnen interagieren.

Siglecs werden hauptsächlich im hämatopoetischen und in Immunzellen exprimiert. Die meisten Siglecs haben ein oder mehrere zytosolische Immunorezeptor-tyrosin-basierende inhibierende Motive (ITIM) in der intrazellulären Domäne, was auf eine Rolle der Siglecs bei Signalkaskaden durch Tyrosin- und Inositolphosphatasen schließen lässt. Diese Zell-Zell-Interaktionen spielen eine wichtige Rolle bei der Aktivierung des Immunsystems und bei der Steuerung von Entzündungsreaktionen. Sie könnten Initiatoren der inflammatorischen Kaskade im Diabetes und des schädlichen Zusammenspiels mit der β-Zelle sein. Daher ist es notwendig, die Rolle der Siglecs im Diabetes zu untersuchen und die möglichen proinflammatorischen Einflüsse, die zur β-Zell-Zerstörung führen, zu verstehen. In dieser Arbeit habe ich die Expression und Funktion der Siglecs im Pankreas und in Immunzellen unter diabetogenen Bedingungen untersucht. In humanen Inselzellen konnte eine zelltypspezifische Expression der Siglecs gezeigt werden: Siglecs-3, -5 und -8 werden von glukagon-produzierenden α-Zellen exprimiert, während Siglecs-1, -2, -7 und -10 in insulin-produzierenden β-Zellen nachgewiesen werden konnten. Die β-zellspezifischen Siglecs-7 und -10 waren im Diabetes niedriger exprimiert, mit gegenteiliger Regulation ihrer Liganden. Die Überexpression von Siglec-7 in β-Zellen konnte deren Funktion verbessern und sie vor Diabetes-induziertem Zelltod schützen, in dem sie den klassischen NF-κB Signalweg und die nachfolgende Zytokinsekretion inhibierte.

Ein ähnlicher Verlust von Siglec-7 wurde auch in aktivierten Monozyten beobachtet. Die Migration von Immunzellen hin zu den entzündeten Inseln konnte ebenfalls durch die Überexpression von Siglec-7 verhindert werden. Zusammengefasst kann man von meinen Beobachtungen darauf schließen, dass Siglec-7 Expression wichtig zur Erhaltung eines antiapoptotischen und überlebensfähigen Status in Inseln ist, und dass dessen Verlust die Aktivierung des Immunsystems und die Inselentzündung verstärkt, was anschließend zu Diabetes führt.

Meine Untersuchungen zeigen erstmalig das Vorkommen von Siglecs in nichtentzündlichen Zellen im pankreatischem Gewebe, was eine komplett neue Perspektive auf die Rolle der Siglecs eröffnet. Diese Arbeit erweitert das Verständnis von entzündlichen Mechanismen in Inseln und zeigt neue Wege auf, um die β-Zellen unter diesen Umständen zu schützen.

1. Abbreviations

ADP	Adenosine diphosphate
AML	Acute myeloid leukaemia
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
APC	Antigen presenting cell
CD	Cluster of differentiation
CKK-8	Cholecystokinin 8
CLSM	Confocal laser scanning microscope
CTL	Cytotoxic T lymphocytes
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
DPP-4	Dipeptidyl peptidase 4
ER	Endoplasmic reticullum
FACS	Fluorescence assorted cell sorting
FADH	Flavin adenine dinucleotide
FADD	Fas associated death domain
FCS	Fetal calf serum
FFA	Free fatty acids
FLIP	FLICE inhibitory protein
Faim/TOSO	Fas apoptotic inhibitory molecule
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GIP	Gastric inhibitory polypeptide
GLP-1/2	Glucagon-like peptide 1/ 2
GlcNAc	*N*-acetylated D-glucosamine
GLUT2	Glucose transporter 2
Gm-CSF	Granulocyte macrophages colony stimulating factor
GSIS	Glucose stimulated insulin secretion
HFD	High fat diet
IFG	Impaired fasting glucose
IFN –γ	Interferon-γ
IL-1β	Interleukin 1β
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based activation motifs
LPS	Lipopolysaccharide
ManNAc	*N*-acetylated D- mannosamine
MCP1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
mRNA	Messenger RNA

NADH	Nicotinamide adenine dinucleotide
Neu3	Neuraminidase 3
Neu5Ac	N-acetylneuraminic acid
NK-cells	Natural killer cells
NOD	Non-obese diabetic
PACAP	Pituitary adenylate cyclase activating polypeptide
PAL	Palmitate
Pax6	Paired box gene 6
PBS	Phosphate buffered saline
Pdx1	Pancreatic and duodenal homeobox 1
PC 1/3	Prohormone convertase 1/3
PC 2	Prohormone convertase 2
PCR	Polymerase chain reaction
PolyI:C	Polyinosinic polycytidilic acid
RNA	Ribonucleic acid
RT-PCR	Real-Time Polymerase chain reaction
ROS	Reactive oxygen species
SHP1/2	SH2 domain-bearing protein tyrosine phosphatase (PTP) 1/2
Sia	Sialic acids
Siglecs	Sialic acid binding immunoglobulin-like lectins
STZ	Streptozotocin
ST8Sia1	α- 2,8 Sialyltransferase 1
TBS	Tris buffered saline
TCA cycle	Tricarboxylic acid cycle
TCR	T cell receptor
TNF-α	Tumor necrosis factor-α
T1D	Type 1 Diabetes mellitus
T2D	Type 2 Diabetes mellitus
WHO	World Health organisation

2. Introduction

2.1 Pancreas: structure and function

The digestive system of vertebrates comprises of gland organs secreting endocrine and exocrine hormones, which facilitate the process of digestion. The pancreas is one of the components of this system and is located between the stomach and the kidneys in higher mammals. In adults, its size ranges from 12.5-15 cm, weighing from 60-100 g [1]. It empties its exocrine digestive juices into the duodenum, while the endocrine secretions are released directly into the blood (Figure 1). The pancreas, in concert with the other digestive organs, is not only responsible for digestion, but also has a prime physiological role in maintaining glucose homeostasis.

Primarily, the exocrine pancreas consists of a network of ducts arising from acinar cells. The acini are arranged at the apices of the branching ductal network, and thus resemble grape clusters. The intralobular ducts create a hierarchical network, forming the pancreatic duct which empties into the duodenum [2]. The digestive juices secreted by the acinar cells comprises of zymogens, precursors of the digestive enzymes, which get cleaved into their active form by enteropeptidases in the intestine. The catabolic enzymes primarily consist of pro-lipases and pro-proteases, aiding the breakdown of ingested lipids and proteins.

Maintenance of normoglycemia in an organism is very crucial for its metabolic balance. In vertebrates, this function is carried out by the endocrine pancreas which consists of the Islets of Langerhans. Named after its discoverer, the German biologist Paul Langerhans, these islets are made up of five different types of cells secreting varied hormones essential for regulation of glucose metabolism: α-cells secreting glucagon, β-cells secreting insulin, δ-cells secreting somatostatin, ε-cells secreting hunger stimulating hormone ghrelin and PP-cells secreting pancreatic polypeptide [3]. In concert with each other, these hormones have several anabolic and catabolic effects in order to keep blood glucose concentration in a narrow range.

2.2 The endocrine pancreas: hormones and their effects

In order to understand the complexity of glucose homeostasis, this chapter introduces the endocrine secretions of the pancreas and their paracrine functions. Somatostatin is an inhibitory peptide, which suppresses the release of several neuronal, gastrointestinal and pancreatic hormones, especially insulin and glucagon [4]. Another hormone secreted post-prandially is the pancreatic polypeptide, whose

action is directed mainly towards suppression of food intake and gastric emptying as well as increase in energy expenditure [5, 6]. The hormone insulin is released by the β-cells in response to several stimuli, the main one being increase in blood glucose concentrations. It ensures clearance of glucose from blood by inducing uptake of glucose by muscle, adipose tissue and liver. It also down-regulates breakdown of stored glycogen (glycogenolysis) so as to prevent rise in blood sugar levels. [7]. On the other hand, when blood glucose levels drop below normal, e.g. after exercise or in-between meals, α-cells produce glucagon which acts mainly on the liver [8]. Glucagon signals drive the hepatic cells to carry out glycogenolysis, thus causing rise in circulating glucose concentrations, ultimately preventing hypoglycemia. Thus, the interplay between insulin and glucagon keeps the plasma glucose concentrations balanced. The secretion of both these hormones is tightly regulated by blood glucose levels and other paracrine signals. To understand the regulation of glucose homeostasis, knowledge of the mechanisms underlying secretion of these hormones need to be addressed.

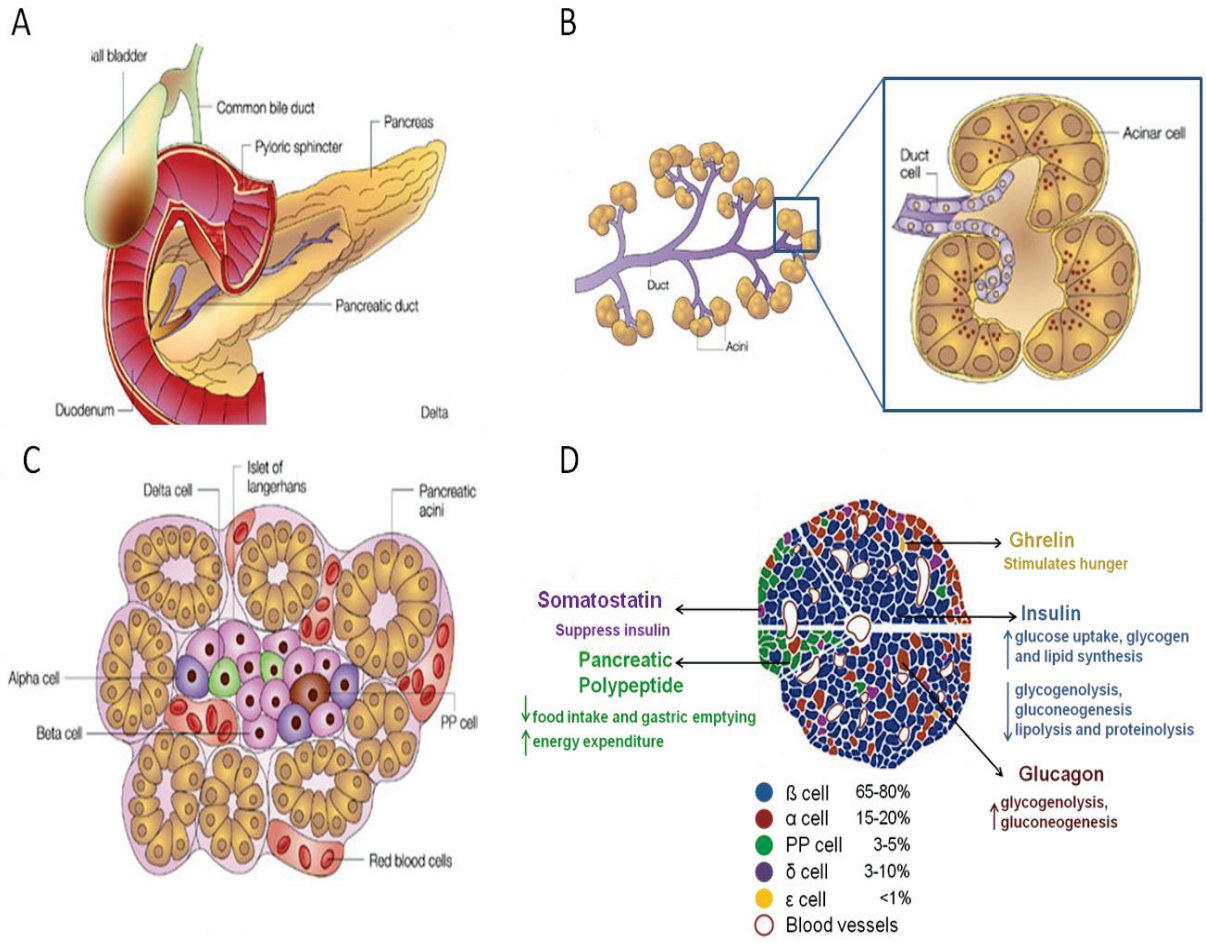

Figure 1: Pancreas: anatomy and physiology. A. Structure of pancreas B. The exocrine pancreas C. The endocrine pancreas D. Structure of Islet of Langerhans. Different cell types and respective hormone production by Islets. (Adapted from [2, 9])

2.3 Insulin: structure and function

Insulin is a peptide hormone, which is synthesized in the β-cells as proinsulin containing a B-, C- and A-chain. Maturation of proinsulin involves proteolytic removal of the C-peptide, thus resulting in a peptide of 51 amino acids consisting of the B- and A-chain bound together by disulphide bonds [10].

2.3.1 Glucose stimulated insulin secretion (GSIS)

In the β-cells, circulating glucose is taken up via the glucose transporter 2 (GLUT2). Subsequent glucose metabolism leads to pyruvate formation and entry into the mitochondrial tricarboxylic acid (TCA) cycle. There is concomitant increase in the cellular ATP:ADP ratio because of electron transfer from the TCA cycle (in the form of NADH and FADH2) to the respiratory chain (oxidative phosphorylation). This

change in the ratio is sensed by the ATP-sensitive potassium (K^+) channels, which shut down in response to this elevation. Thus, membrane depolarization takes place, which results in Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. The Ca^{2+} pulses trigger insulin granule exocytosis, and the insulin granules are released directly into the blood (Figure 2). Insulin circulates through the body and acts on different organs, including muscles, adipose tissues and liver. To ensure glucose clearance, insulin not only promotes glucose uptake, glycogen and lipid synthesis, but also inhibits glycogenolysis, synthesis of glucose from precursors (gluconeogenesis), breakdown of lipids and proteins (lipolysis and proteinolysis).

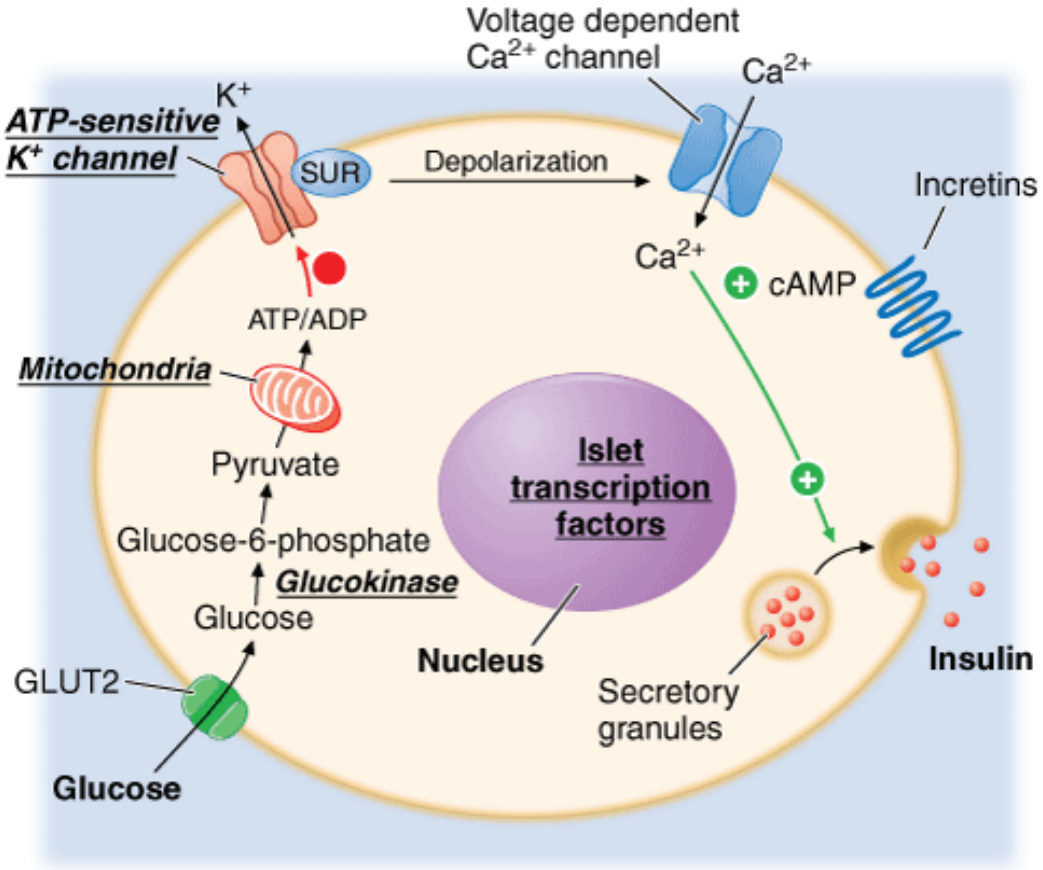

Figure 2: Mechanism of insulin secretion by the β-cells [11]. Uptake and metabolism of glucose leads to elevation in the ATP: ADP ratio, which causes the potassium channels to shut, leading to membrane depolarization and influx of calcium which aids in the exocytotic release of insulin from its storage granules. (Adapted from[11])

2.3.2 Incretins: stimuli for insulin secretion

As compared to intravenous administration of glucose, higher levels of insulin are secreted by β-cells upon oral intake of glucose. This potentiating effect of glucose

stimulated insulin secretion has been attributed to existence of gut-derived factors, called incretins, and therefore referred to as the 'incretin effect'. Glucose-dependent insulinotropic peptide (GIP) was the first incretin to be characterized which can potentiate GSIS [12]. Nevertheless, complete removal of GIP does not terminate this gut-mediated potentiation. This observation hinted towards the presence of additional factors contributing in this process. A second peptide with incretin activity was identified more than a decade after the discovery of GIP, by characterization and cloning of the proglucagon gene. In both preclinical and human studies, the ability of Glucagon-like peptide-1 (GLP-1), a peptide co-encoded carboxyterminally to glucagon in the proglucagon gene, to potentiate GSIS was demonstrated [13, 14]. Intestinal L-cells secrete GLP1 following nutrient intake [14]. Similar to glucose, GLP-1 contributes to closure of ATP-sensitive K (K_{ATP}) channels, aiding in membrane depolarization and the induction of electrical activity (Figure 3). Upon exposure to elevated levels of glucose and GLP-1, Ca^{2+} influx is induced, accompanied by mobilization of intracellular Ca^{2+} stores through protein kinase A (PKA) and exchange protein activated by cAMP (EPAC2)-dependent mechanisms. As a consequence, insulin exocytosis is triggered and further potentiated by increased cAMP levels. Owing to the binding of cAMP to PKA and EPAC2, there is accelerated insulin granule mobilization, which amplifies the insulin pool that is available for immediate release [15]. Hence, GLP1 has an effect on "first phase" of insulin secretion wherein stored insulin granules are rapidly released as a response to elevated glucose levels.
In spite of the expanding knowledge on mechanisms and regulation of insulin secretion from the β-cells, the phenomenon of glucagon secretion by the α-cells in response to glucose is not as well understood. Both, paracrine signals [16] and intrinsic regulation [17], are under debate, and it remains unclear whether glucagon secretion is primarily modulated by any or both of these mechanisms.

2.4 Glucagon: structure, secretion and function

Glucagon is a hormone secreted by α-cells in the Islets of Langerhans. Being a member of the pituitary adenylate cyclase activating polypeptide (PACAP) superfamily of peptide hormones, proglucagon gene is expressed as a 2-kb transcript encoding glucagon and glucagon-like peptides (GLP-1 and GLP-2). Prohormone convertase 1/3 (PC3) processes proglucagon to form GLP-1 and GLP-2 in the L-cells of the duodenum.

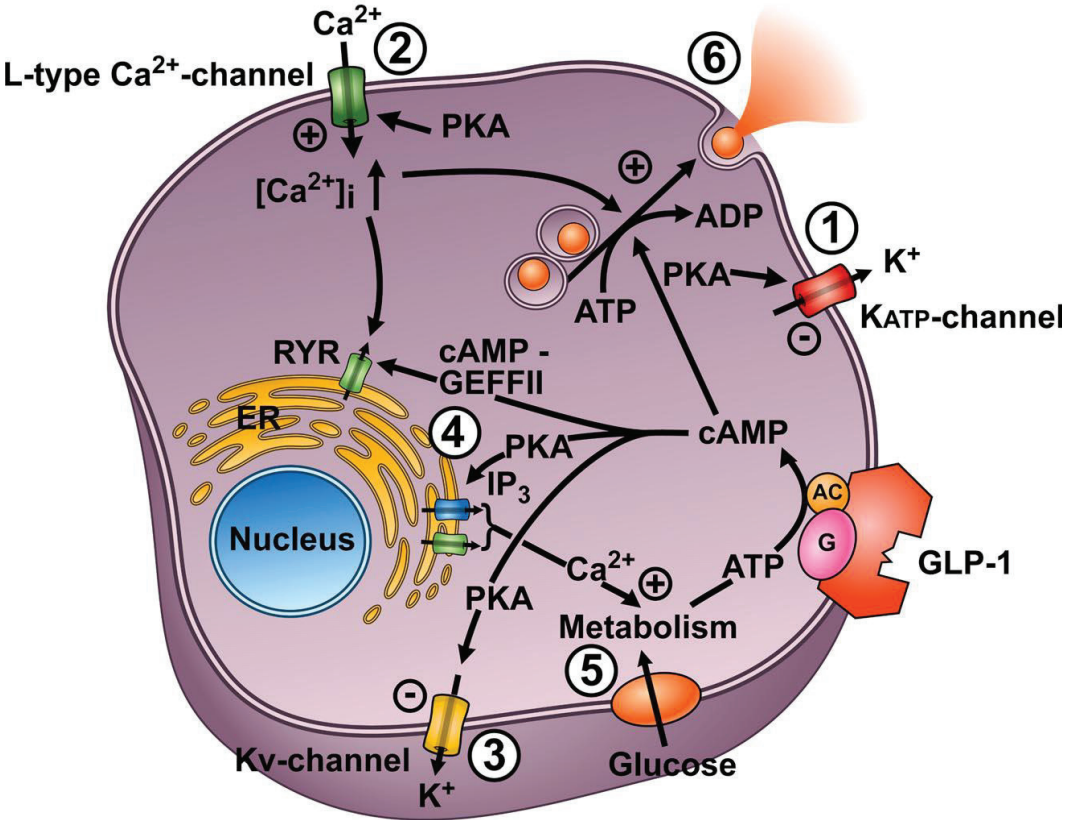

Figure 3. GLP-1 potentiates the stimulation of insulin secretion [18]. GLP-1 binds to its receptor on the β-cell, which results in activation of adenylate cyclase. As a consequence, intracellular cAMP levels are increased, followed by activation of protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII, also known as EPAC2). The potentiating effect comes from the ability of cAMP to accelerate insulin granule mobilization, and thus causes an increased amount of readily available pools of granules which can be instantly released. [Modified from Holst and Gromada [19]].

Prohormone convertase 2 (PC2) expressed in the pancreatic α-cells, processes the proglucagon into glucagon, which is a single 29 amino acids long polypeptide chain with a molecular weight of 3483 dalton [20],[21],[22]. In response to elevated glucose concentrations, glucagon secretion is down-regulated. Investigations for underlying mechanisms of this regulation have shown various regulatory molecules like Zn^{2+} or γ-Aminobutyric acid (GABA) secreted by the β-cells [23] and somatostatin released by neighboring δ-cells [24] to be able to inhibit glucagon secretion. In spite of the neuronal regulation of glucagon secretion, an additional paracrine effect of products secreted by β-cells in face of hyperglycemia play a primary role [25, 26].

Glucagon carries out its primary function of glucose homeostasis through its actions on the liver. The main impact of glucagon in liver is an increase in hepatic glucose output, which comes from increase in glycogenolysis, gluconeogenesis, and reduction in glycolysis. In T2D, with subsequent insulin insufficiency, the regulation of glucagon secretion is lost; and hyperglycemia induces glucagon secretion, instead of inhibiting it, thus causing further disruption of the insulin-glucagon axis [26, 27].
The balanced and regulated secretion of these endocrine hormones is thus essential for maintaining blood glucose levels. To summarize the previous chapters, the apparent dysregulation in insulin and glucagon production and secretion in the endocrine pancreas, as well as signaling in the insulin-responsive tissues, leads to the metabolic syndrome: diabetes.

2.5 Diabetes: Incidence and Etiology

Diabetes mellitus, or diabetes, is a metabolic syndrome in which regulation of glucose metabolism is lost, leading to abnormally high plasma glucose concentrations. Its incidence is attributed to complex events arising from a combination of hereditary and environmental causes. The current generation has seen an enormous increase in the number of individuals suffering from diabetes, with more than 366 million people being diabetic, as per the WHO factsheet. WHO also projects that diabetes will be the 7th leading cause of death in 2030 [28]. To curb this epidemic and introduce new preventative and curative therapies, clear understanding of the molecular mechanisms leading to occurrence of diabetes needs to be prioritized. Some of the medical conditions associated with diabetes are: high blood pressure, elevated cholesterol levels, coronary artery disease, post gestational diabetes, polycystic ovary syndrome, chronic pancreatitis, fatty liver, hemochromatosis, cystic fibrosis, several mitochondrial neuropathies and myopathies, myotonic dystrophy, Friedreich's ataxia, some of the inherited forms of neonatal hyperinsulinism [29]. Diabetes-related complications are varied and are even fatal. When medications are not able to bring diabetes under control, acute complications (hypoglycemia, ketoacidosis, or nonketotic hyperosmolar coma) may occur [30]. Prolonged imbalances in glucose homeostasis may result in secondary complications like cardiovascular disease (doubled risk), chronic renal failure, retinal damage (which can lead to blindness), nerve damage (of several kinds), and microvascular damage, which may cause erectile dysfunction and poor wound

healing [30]. Lifestyle interventions such as proper diet, regular exercise, blood pressure control and non-smoking may improve susceptibility to various complications. Diabetes manifests itself in the form of three classical symptoms: frequent urination or polyuria, increased fluid intake or polydipsia and increased appetite or polyphagia [31]. Depending on the primary cause of incidence, diabetes is divided into two major types, viz. type 1 diabetes (T1D) (formerly known as childhood-onset or insulin-dependent diabetes) and type 2 diabetes (T2D) (formerly known as adult-onset or non-insulin dependent diabetes) [31].

2.5.1 Type 1 Diabetes mellitus (T1D)

T1D incidence is majorly attributed to the autoimmune destruction of the insulin producing β-cells, resulting in an absolute insulin deficiency [32]. As is true in most of the autoimmune disorders, interactions between environmental factors and genetic predispositions contribute to the triggering of T1D [33, 34]. The manifestation and progression of T1D can be divided into two phases: a moderate to long-term symptomless phase of insulitis wherein islets are infiltrated by a mixed population of leukocytes [35] and overt diabetes with evident hyperglycemia and hypoinsulinemia where most β-cells are destroyed. Study of various T1D mouse models, typically non-obese diabetic (NOD) mice, have given insights into mechanisms of onset and development of T1D [36]. Regardless of the interplay of various immune cells like B-cells, macrophages and dendritic cells being responsible for T1D development, it is considered to primarily be a T-cell mediated disease. Activation of the cytotoxic T-cell response has been observed against several autoantigens including peptides derived from insulin [37] and glutamic acid decarboxylase (GAD) [38]. The initial step in the development of T1D is thought to be autoantigen presentation by macrophages and/ or dendritic cells to CD4+ T-helper cells. Subsequent secretion of various cytokines including interleukin (IL) -1β, tumor necrosis factor (TNF) -α and interferon (IFN) -γ by the activated macrophages and CD4+ T-cells induce CD8+ T-cell activation and infiltration in the islets. These cells then destroy β-cells by different mechanisms such as release of granzymes and perforin as well as by Fas - Fas-Ligand interactions [39] (Figure 4). Triggers for β-cell specific destruction vary leading to variable clinical parameters in patients. Therefore, diverse phenomenon could initiate the incidence of T1D, involving different initiating antigens, primary effector cell types and mechanisms of β-cell death [35]. A major therapeutic intervention for T1D is islet

transplantation, but graph rejections and post transplantation complications are a crucial risk factor. Inflamed state of the transplanted islets as observed by increased secretion of monocyte-chemoattractant protein-1 (CCL2/MCP-1) and tissue factor (TF) tends to negatively contribute to graft function and outcome [40].

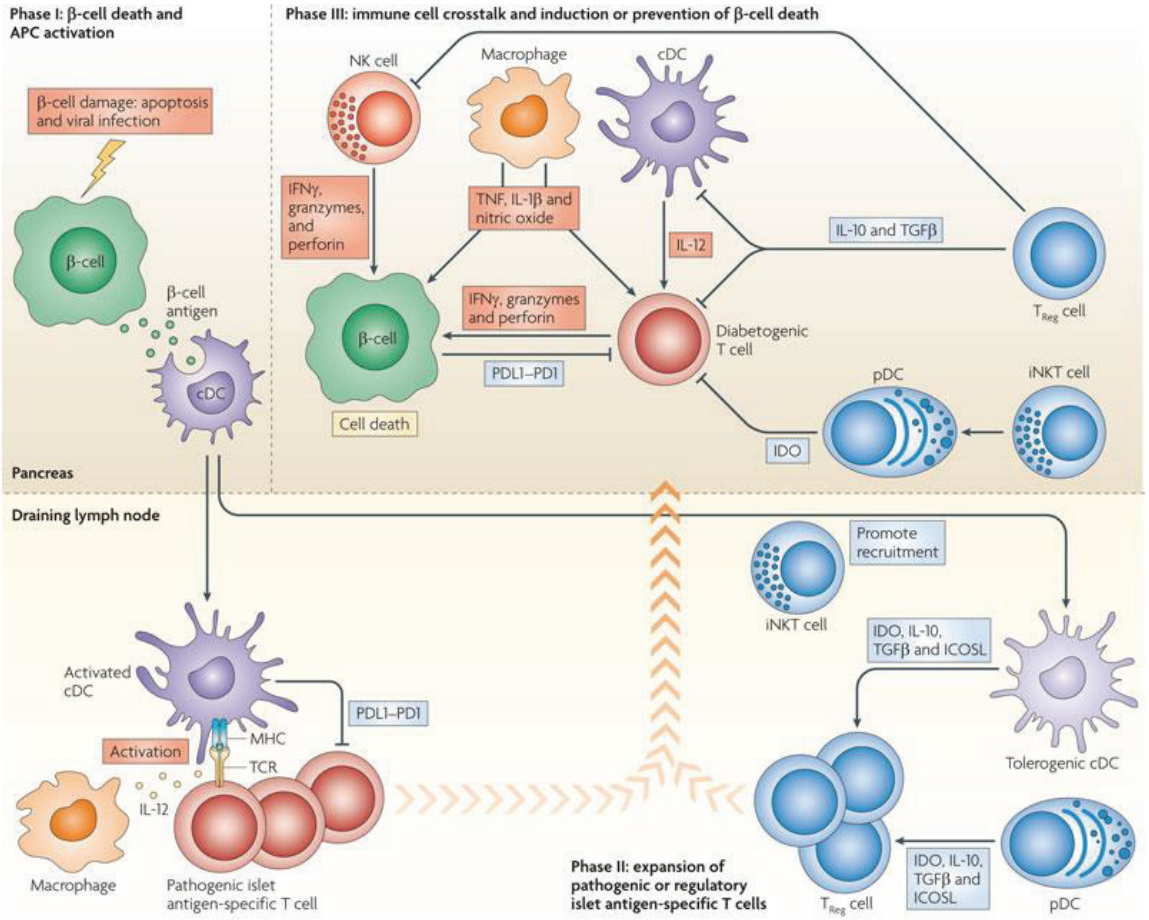

Figure 4. Cellular and molecular mechanisms in the development or prevention of type 1 diabetes [41]. In the initiation phase of T1D following β-cell damage, conventional dendritic cells (cDCs) capture and process β-cell antigens. Phase 2 is marked by the migration of activated cDCs to the lymph nodes where they prime pathogenic islet antigen-specific T cells. In Phase 3, β-cells can be killed by diabetogenic T cells and NK cells through the release of interferon-γ (IFNγ), granzymes and perforin, as well as by macrophages through the production of tumour necrosis factor (TNF), IL-1β and nitric oxide. This complex crosstalk between innate and adaptive immune cells results in the development of T1D. APC, antigen-presenting cell; TCR, T cell receptor.(Adapted from **[38]**)

2.5.2 Type 2 Diabetes mellitus (T2D)

T2D manifests itself as a progressive decline in β-cell function along with chronic insulin resistance. The development of T2D is strongly affected by environmental factors, such as sedentary lifestyle, nutrition and obesity.

Nevertheless, only a small percentage of obese people suffer from diabetes, and non-obese diabetic individuals also exist, hinting that other factors are also involved in the etiology of T2D. Genetic predisposition is also one of the parameters affecting the incidence as evidenced by association of polymorphisms within many genes with decreased/increased risk of T2D [42]. Disease progression in T2D has been proposed to have five stages [43]: Stage 1 is compensatory increase in insulin secretion in the face of insulin resistance. This compensation has been attributed to both expansion of β-cell mass [44, 45] and increased β-cell function [46, 47]. Stage 2 is a static state of β-cell adaptation that can last for years but is already accompanied by loss of β-cell mass and acute glucose-stimulated insulin secretion [48]. Stage 3 is an unstable, transient stage wherein the compensation starts falling short of the demand for insulin and glucose levels start rising, leading to stage 4 of fulminate diabetes. In most cases, this stage lasts for the entire lifespan of a patient as they typically have enough insulin secretion left rather than progressing to ketoacidosis. Only in T1D does the disease progress to Stage 5 where severe destruction of β-cells leads to ketoacidosis and the patient becomes dependant on insulin administration for survival.

2.6 Insulin resistance

In contrast to T1D, where severe destruction of the islets drastically reduces the supply of the hormone insulin, in T2D there is insulin production at diagnosis, and often even hyperinsulinemia. However, the insulin is insufficient in stimulating glucose clearance by target tissues. Insulin resistance is the condition in which the biological effects of insulin are less pronounced for both glucose disposal in skeletal muscle and inhibition of gluconeogenesis in the liver [49]. Healthy individuals compensate for this insulin resistance by increasing insulin secretion by increase in β-cell mass and function. However, in about 10% of individuals, this compensation fails to meet the metabolic demand, hence resulting in chronic hyperglycemia and diabetes [50]. Inflammation of multiple organ systems like liver, muscle, adipose tissue and hypothalamus have been associated with insulin resistance. The loss of

energy homeostasis due to hepatic inflammation occurs through decreased hepatic glycogen storage, increased gluconeogenesis, and lipogenesis, associated with hepatic lipid accumulation [51]. Hypothalamic inflammation disrupts leptin and insulin signaling [51, 52]. Also, inflammation inhibits muscle glucose uptake and glycogen synthesis [51, 52]. Adipose tissue inflammation has been implicated during the course of development of insulin resistance, with amplification of the pro-inflammatory environment by secretion of adipokines and cytokines [53].

The phenomenon of insulin resistance results in increased demand for insulin to maintain normoglycemia, which stresses the pancreatic β-cells to increase insulin secretion or its mass [54]. When these compensatory mechanisms fail to meet the increased demand of insulin, glucose homeostasis is disturbed and T2D develops [54].

2.7 Defects in incretin mediated insulin secretion

T2D is essentially a plethora of regulatory malfunctions. One of the aspects of T2D has been the abnormalities in incretin-mediated insulin secretion [55], in the form of defective GIP action and reduced GLP-1 secretion and action [56]. Loss of glucose homeostasis observed in T2D, thus, is partially resulting from the decreased incretin effect [57]. When the secretion of GIP and GLP1 in response to elevated glucose was compared in both normal subjects and patients with T2D, only healthy subjects were able to respond to orally administered glucose, by increasing its insulinotropic effect [58]. Also, in patients with T2D, postprandial GIP responses were less affected than the GLP-1 response [59]. In addition to this, the defective incretin action can also impair the suppression of glucagon secretion in response to nutrient intake, thus further contributing to the endocrine hormonal imbalance observed in T2D [60, 61].

A crucial factor contributing to the short-term stimulatory effect of the incretins is their degradation and inactivation by enzymes which maintain their low half-life in serum. Dipeptidyl peptidase 4 (DPP-4) is a ubiquitous cell membrane associated enzyme responsible for cleaving and inactivating both incretins: GLP-1 and GIP [62] (Figure 5). Thus, some strategies to restore the incretin effect in T2D are: use of injectable GLP-1 receptor agonists, or use of DPP-4 inhibitors to increase half-life of endogenous GLP-1. These treatments have been shown to be effective in stimulating insulin secretion as well as in inhibition of glucagon secretion, without any risk for hypoglycaemia [63], although an increased risk for pancreatitis has been recently

discussed [64]. Even though long-term clinical effects of incretin-based drugs on the β-cell are still speculative [65], numerous *in vivo* and *in vitro* studies in rodents show that GLP-1 increases β-cell mass, proliferation [66-68] and β-cell neogenesis [69]. GLP-1 has been shown to not only inhibit β-cell apoptosis in human islets [70-72], but also has a protective role in islets isolated from patients with T2D [73]. On these lines stabilizing GLP-1 by DPP-4 inhibition restores glycemic control in diabetic animal models [74], improves β-cell survival, replication and neogenesis in the diabetic mouse and rat streptozotocin model [75, 76] and in the VDF diabetic rat [77].

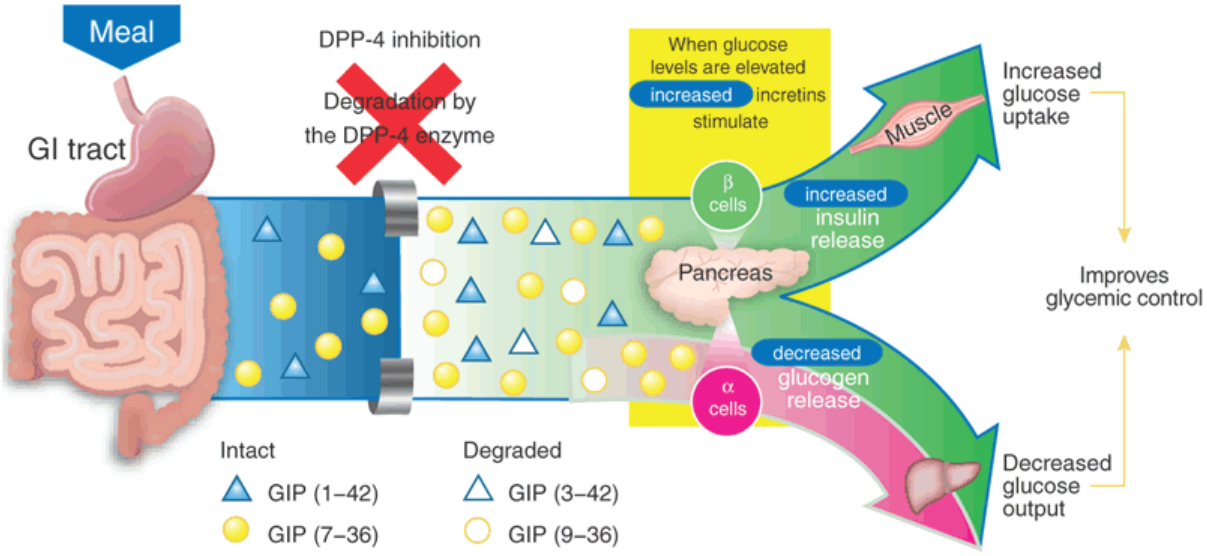

Figure 5: The incretin effect on glycemic control (adapted from [78]) The oral ingestion of glucose leads to secretion of incretins GLP1 and GIP, which potentiate insulin secretion by the β-cells and inhibit glucagon secretion by the α-cells. Periodically, DPP-4 cleaves off the incretins, so as to control this potentiation. DPP-4 inhibitors help to maintain the incretins in circulation for longer durations, thus helping glucose clearance in diabetic individuals.

Nevertheless, the direct effect of DPP-4 inhibitors on human β-cells has not been investigated yet, which has been addressed in this thesis.

2.8 Decreased β-cell function and mass

As discussed earlier, T2D is characterized by progressive β-cell dysfunction which begins as a loss in first-phase insulin secretion and followed by reduction of glucose sensitivity of second-phase insulin secretion, that leads to subsequent β-cell exhaustion, culminating in reduction of β-cell mass [79, 80]. The symphony of events contributing to defective insulin secretion in T2D can be summarized as impaired

glucose stimulus-secretion coupling, altered insulin granule exocytosis and reduction in insulin biosynthesis [80]. In addition to these secretory defects, the continual stress on the β-cells in T2D leads to loss of β-cell mass, when compared to age- and weight-matched non-diabetic controls [80]. β-cell mass is plastic and fluctuates to maintain a balance between insulin supply and demand. β-cell compensation is the term coined to define the adapted increase in β-cell mass upon increased metabolic load caused by obesity and the inherent insulin resistance. In only 10% of pre-diabetic individuals this compensation is inadequate leading to manifestation of T2D [50]. As seen by a study investigating human pancreata from autopsy, patients with impaired fasting glucose (IFG) revealed a 40% loss of β-cell mass, whereas in T2D, 63% of β-cell mass was lost as compared to weight matched controls [54]. In addition to this, lean diabetic patients had loss of 41% of β-cell volume compared with lean controls. This decreased β-cell mass was found correlated with increased β-cell apoptosis. The observed loss in β-cell mass in pre-diabetic individuals with impaired fasting glucose levels provides evidence for β-cell destruction even at early stages of T2D [54]. Diverse phenomena like endoplasmic reticulum (ER) stress, oxidative stress, chronic hyperglycemia, dyslipidemia, and systemically and locally increased levels of cytokines, have been implicated to trigger β-cell apoptosis occurring during the pathogenesis of T2D.

2.9 Mechanisms of β-cell death

In T1D, destruction of β-cells occurs through activation of immune mediated processes such as mononuclear cell infiltration in the pancreatic islets; interaction between antigen presenting cells and T-cells which leads to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other inflammatory products [81, 82]. Such cytokines and chemokines secreted by various cell-types like activated macrophages, adipocytes and also by pancreatic β-cells have been suggested to trigger β-cell apoptosis [83]. Two major pathways have been thought to occur at the onset of T1D; the perforin/granzyme and the Fas/FasL system [35]. Focusing on the Fas/FasL system, the expression of Fas and Fas ligand has been observed in inflamed islets in pancreas sections of patients with recent-onset T1D [84] as well as in patients with poorly controlled T2D [85].

The Fas receptor (CD95), a 45-kDa type I trans-membrane protein, is activated by interactions between β-cells and T-cells. It can also be activated through expression

of inflammatory mediators, i.e., cytokines, chemokines and other inflammatory compounds [35, 86], in addition to its binding to the endogenous ligand (FasL). The engagement of Fas-FasL triggers a death signaling cascade in the β-cells [86].

FasL is a type II transmembrane protein of 40 kDa which is secreted in a soluble form (26 kDa), and is primarily expressed on activated T-lymphocytes [87] and also on β-cells [85, 88]. Upon activation, Fas-associated death domain protein (FADD) and caspase-8 (also known as FLICE) is recruited to the receptor. Following caspase-8 recruitment, there occurs an autoproteolytic cleavage which activates it, and there is subsequent release of the active subunits. A new cycle of activation starts when the remaining caspase-8 prodomain is replaced by uncleaved procaspase-8. Previously our lab has shown that exposure of the islets to elevated glucose concentrations induced Fas expression, caspase-8 and -3 activation, and β-cell apoptosis [85]. Additional investigations revealed that, Fas signals do not always result in apoptosis but can also trigger a pathway that leads to proliferation [89]. An inhibitor of Fas-induced apoptosis, termed cellular FLICE (caspase-8)-inhibitory protein (FLIP) [90] structurally resembles caspase-8 and thus interferes with its recruitment to the death-inducing signaling complex (DISC) (Figure 6). Thereby, FLIP can act as a switch capable of turning apoptotic signals into pro-survival ones [91].

We have previously investigated this regulatory ability of FLIP with regards to its potential to expand β-cell mass [92]. We observed a drastic decrease in endogenous FLIP levels upon exposure of human islets to elevated glucose concentrations. Restoring the FLIP levels in these islets by over-expression induced an increase in β-cell proliferation and reversal of apoptosis. In support of our work, a study by Cottet *et al.* has shown that FLIP is also protective against cytokine-induced activation of caspase-8-dependent apoptosis in an insulin-secreting cell line [93].

An upstream regulator in the Fas signaling is the cell surface protein TOSO or Fas apoptotic inhibitory protein (Faim). It has been described to be expressed in activated T-cells [94, 95]. In Jurkat cells, TOSO has been shown to protect from Fas induced apoptosis through expression of FLIP [94]. Also, in TOSO deficient mice, FLIP expression is down-regulated, rendering these mice to be susceptible to Fas triggered apoptosis [96]. In spite of the accumulating evidences of the anti-apoptotic role of TOSO, its presence and function in the pancreas remains to be elucidated. Such an investigation would provide a promising tool to block Fas induced apoptosis observed in β-cells during the manifestation of diabetes. Taking this into

consideration, this thesis addresses the presence and function of TOSO in the pancreatic islets, and brings forth novel observations on its role in blocking the Fas-receptor mediated death signaling in these cells.

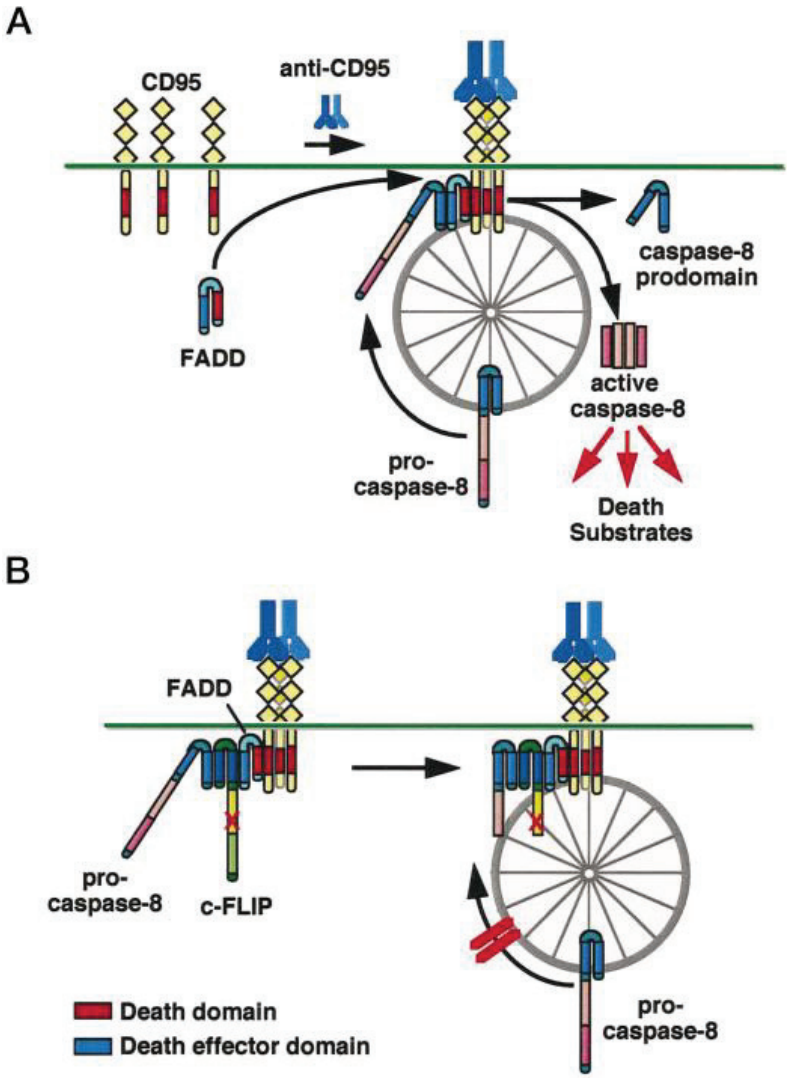

Figure 6. Model for c-FLIP-mediated resistance to CD95 (Fas)-induced apoptosis [97]. A. Anti-CD95 mAb triggers CD95 leads to the recruitment of FADD and caspase-8 to the receptor, followed by autoproteolytic cleavage and activation of caspase-8. The released pro-domain is replaced by uncleaved procaspase-8, thus initiating another round of activation. B. This progression can be blocked by recruitment of c-FLIP along with caspase-8. Following initial cleavage of both molecules, the cleavage intermediates remain bound to the receptor and can no longer be replaced by procaspase-8. (Adapted from [97])

On a broader note, β-cell toxicity has been linked to various stimuli including elevated glucose, free fatty acids and, pro-inflammatory cytokines, leading to ER stress, and oxidative stress (Figure 8) [98-101]. Elevated glucose concentrations can induce β-cell death and this may be a crucial mechanism causing β-cell failure in diabetes.

High glucose concentrations are detrimental to β-cell function and survival in multiple ways such as induction of oxidative and ER stress, β-cell overstimulation and elevated levels of Ca2+, inflammation, hexoamine pathway and β-cell de-differentiation [100, 102, 103]. Reports of glucose-induced β-cell apoptosis have been observed in several animal models of T2D including the *Psammomys obesus* or the ZDF rat [104, 105]. Not only glucotoxicity, but also lipotoxicity plays an important role in underlying mechanism of type 2 diabetes. Chronically elevated levels of FFAs like palmitic acid induces β-cell apoptosis via varied mechanisms like generation of ceramide and ROS and ER stress [106-108]. FFAs, in combination with elevated glucose levels, further induce β-cell apoptosis, termed as glucolipotoxicity [109]. Another way in which this diabetogenic milieu can bring about initiation and/or progression of T2D is the triggering of chronic inflammation.

2.10 Systemic and pancreatic inflammation

Chronic low-grade inflammation has been shown to precede and predict the development of T2D [110]. Sustained activation of the immune system exists in obesity as witnessed by adipose tissue macrophage infiltration and pro-inflammatory activity of macrophages [111]. Both these cell-types have been proposed to be the origin of elevated circulating levels of various cytokines and chemokines, such as IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and TNF-α observed in patients with T2D [111]. Elevated levels of circulating pro-inflammatory cytokines have been implicated in development of insulin resistance [112], loss of β-cell function [113]. Application of anti-inflammatory mediators may reverse both these deleterious effects [114, 115], re-iterating the role of inflammation in development of T2D (Figure 7).

Inflammation is local response of the immune system which involves immune cell migration, release of cytokines and chemokines leading to functional or structural damage of the invaded tissues. Inflammation is the red flag that locates and eliminates any infections, and also promotes regeneration of the damaged tissue. in spite of this, a by-stander effect might amplify the tissue damage due to inflammatory mediators, reactive oxygen species, and complement components [116]. When looked at islets from T2D individuals, the occurrence of inflammation is evidenced by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits, and eventually fibrosis in these islets.

As the presence of inflammatory cells in islets in T2D has only recently been shown, inflammation of pancreatic islet in T2D is a recent concept in the context of T2D. This immune cell infiltration has been demonstrated in several animal models of T2D and in human pancreatic sections of patients with T2D [117, 118]. High fat diet increased the number of islet-associated $Cd11b^{+}$ cells in C57BL/6 mice as early as after 8 weeks on this diet. Similar induction is also seen in the spontaneous model of T2D, the leptin receptor deficient db/db mouse. As compared to non-diabetic controls, an increased number of $CD68^{+}$ cells/islet are seen in patients with T2D. The activated status of macrophages was indicated by expression of HLA-2 and CD163 by these cells [117]. This hints towards the possibility of inflammation being one of the triggering events in T2D pathophysiology and which probably also contributes to the progression of the disease. Thus, in addition to systemic tissue inflammation, local islet inflammation might precede incidence of T2D.

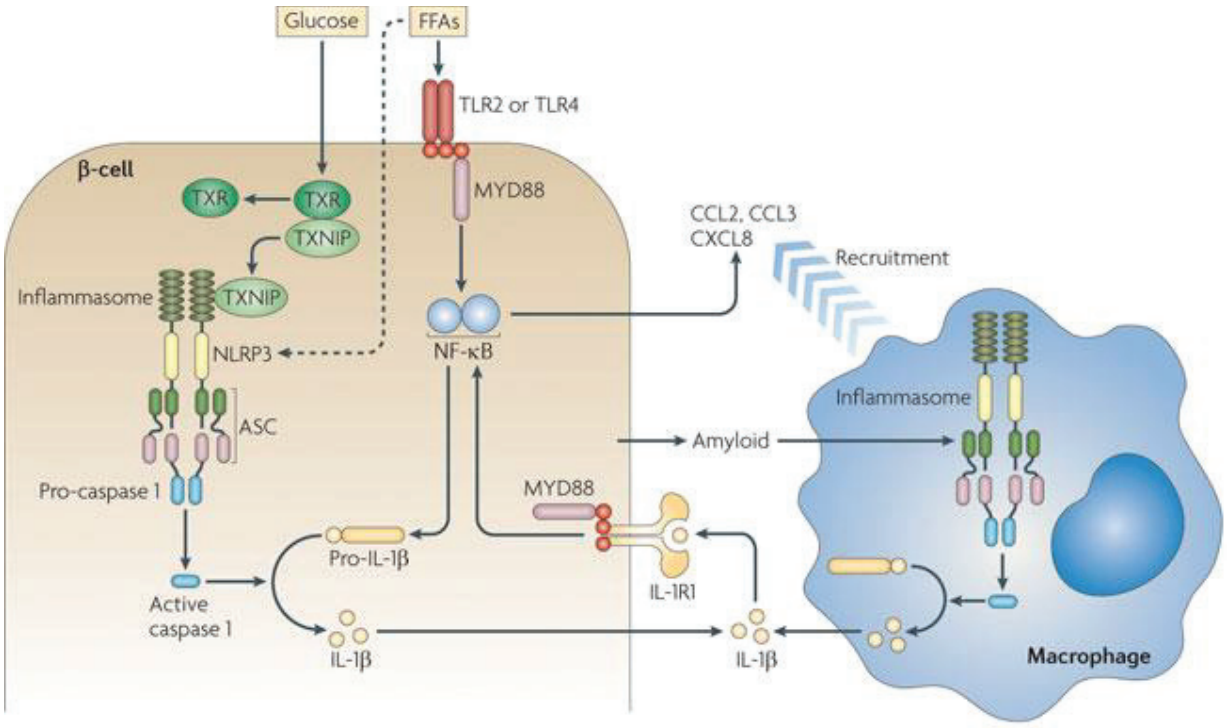

Figure 7. Type 2 diabetes as an inflammatory disease[99]. Elevated glucose levels induce production of interleukin-1β (IL-1β) by the β-cells, through the dissociation of thioredoxin-interacting protein (TXNIP) from its inhibitor thioredoxin (TXR), which results in activation of the NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome, activation of caspase 1 and processing of pro-IL-1β into its mature form. By activating nuclear factor-κB (NF-κB), IL-1β induces the production of a wide range of cytokines and chemokines such as CC-chemokine ligand 2 (CCL2),

CCL3 and CXC-chemokine ligand 8 (CXCL8). This is enhanced by free fatty acid (FFA)-induced activation of Toll-like receptor 2 (TLR2) or TLR4 and leads to the recruitment of macrophages. FFAs may also directly activate the NLRP3 inflammasome. Islet-derived amyloid can activate the recruited macrophages through the NLRP3 inflammasome, increasing IL-1β production and the vicious cycle of IL-1β autostimulation through IL-1 receptor type 1 (IL-1R1). ASC, apoptosis-associated speck-like protein containing a CARD; MYD88, myeloid differentiation primary-response protein 88. (Adapted from [99])

2.8.1 Cell-Cell interactions in Inflammation

In the event of activation of an inflammatory response, elaborate series of events take place, which culminate in the accumulation of leukocytes and other mediators in the inflamed tissue. Various cell types of the immune system interact with each other and these interactions lead to the manifestation of the symptoms observed in inflammation. Of these leukocytes, the cell types that are circulating in the blood are neutrophils, monocytes, eosinophils, lymphocytes, basophils. Only the lymphocytes have the ability to show diversity, specificity, memory, and self/non-self recognition, the hallmarks of an adaptive immune response. The other cell types assist in this adaptive immune response by either activating these lymphocytes, regulating antigen clearance or secreting various immune mediators. Differential expression of membrane proteins is used to identify different lineages or maturational stages of lymphocytes. These cell surface proteins are termed as a cluster of differentiation (CD) which are used in defining the sub-populations of these cells [119]. These cell surface molecules play an important role in the functional interactions between cells of the immune system. Along with cell surface receptors, cell adhesion molecules also contribute to the activation and function of the cell. Various adhesion molecules have been shown to contribute to the interactions between helper T(T_H) cells and APCs, T_H and B cells, and cytotoxic T lymphocytes (CTLs) and target cells. One such adhesion and signaling molecules are Sialic acid binding immunoglobulin-like lectins (Siglecs).

2.11 Sialic acids

Glycocalyx of a cell is decorated with glyco-conjugates which are contributing to the ability of a cell to sense the external conditions. Sialic acids (Sia) are the most abundant terminal carbohydrate motifs on cell surface glyco-conjugates in eukaryotic

cells. Sialic acids (N-acetylneuraminic acid (Neu5Ac)) are a family of acidic 9-carbon amino sugars (Figure 8). Neu5Ac, is synthesized in the cytoplasm from *N*-acetylated D-glucosamine (GlcNAc) or D-Mannosamine (ManNAc) by several sequential enzymatic reactions [120]. Neu5Ac gets converted to monophosphate (CMP)-activated Neu5Ac in the nucleus [121], which is then transferred onto oligosaccharides of glycoconjugates by specific sialyl transferases in the Golgi apparatus [122]. In nature more than 40 forms of sialic acids exist due to additional modifications like *O*-glycosylation [123]. In addition, sialic acids can be attached in a variety of linkages to the underlying glycans on *N*-glycans, *O*-glycans or glycolipids, which are responsible for their diversity and specificity [124].

Sialic acids are known to play crucial roles in many cell-cell interactions, which can be attributed to their prominent position, negative charge and widespread distribution [123, 125] (Figure 8). As a consequence, altered sialylation patterns have also been shown to affect cellular responses such as tumorigenicity and metastatic behavior of malignant cells [126, 127]. Taking into account their role in cellular communication, the discovery of a group of lectins that specifically recognize sialic acids, i.e siglecs, expressed on mammalian cells of the hematopoietic system has given rise to a new perspectives of their role in regulation of immune response.

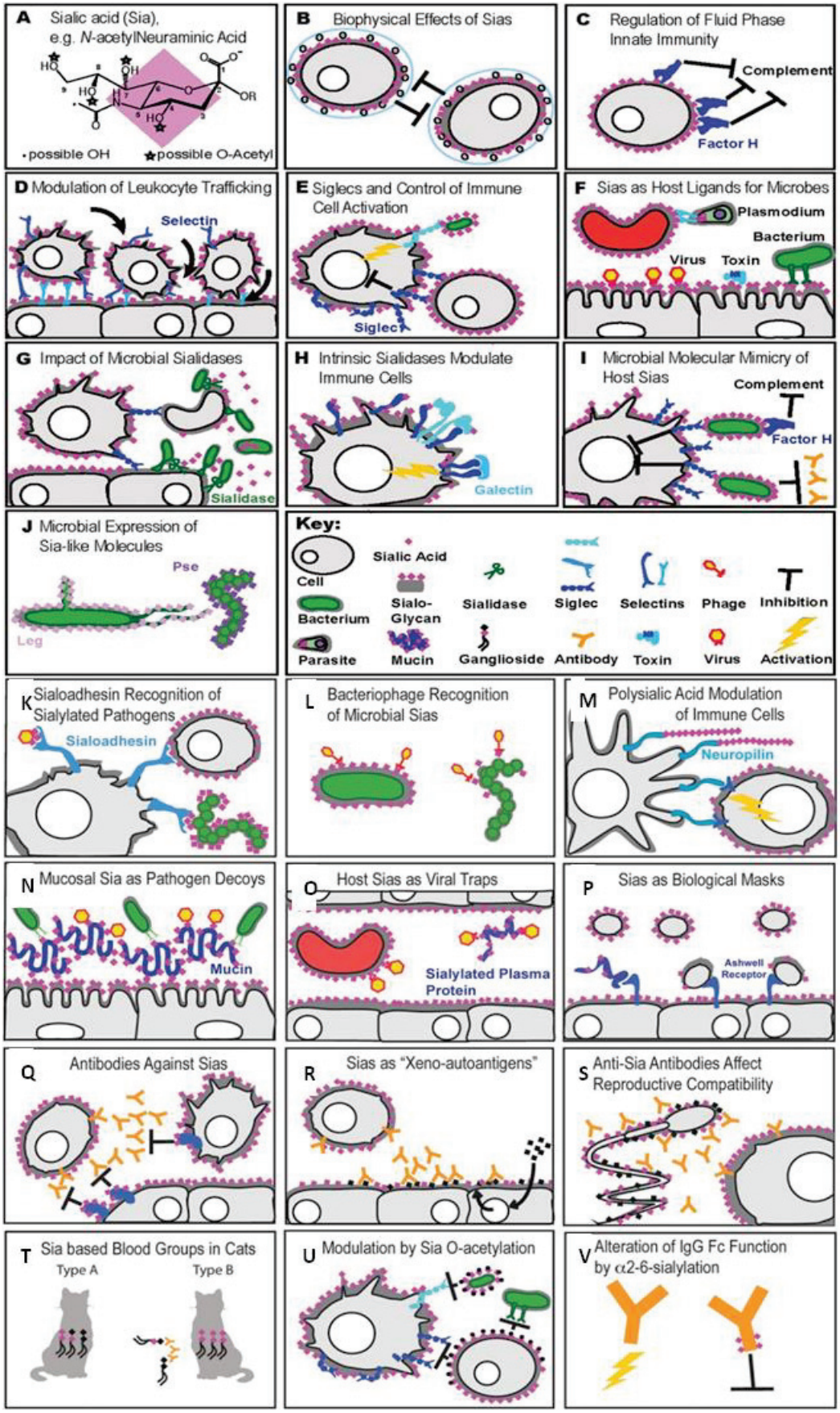
A Sialic acid (Sia), e.g. N-acetylNeuraminic Acid
possible OH
possible O-Acetyl
B Biophysical Effects of Sias
C Regulation of Fluid Phase Innate Immunity
Complement
Factor H
D Modulation of Leukocyte Trafficking
Selectin
E Siglecs and Control of Immune Cell Activation
Siglec
F Sias as Host Ligands for Microbes
Plasmodium
Bacterium
Virus
Toxin
G Impact of Microbial Sialidases
Sialidase
H Intrinsic Sialidases Modulate Immune Cells
Galectin
I Microbial Molecular Mimicry of Host Sias
Complement
Factor H
J Microbial Expression of Sia-like Molecules
Pse
Leg
Key:
Cell
Sialic Acid
Bacterium
Sialo-Glycan
Sialidase
Siglec
Selectins
Phage
Inhibition
Parasite
Mucin
Ganglioside
Antibody
Toxin
Virus
Activation
K Sialoadhesin Recognition of Sialylated Pathogens
Sialoadhesin
L Bacteriophage Recognition of Microbial Sias
M Polysialic Acid Modulation of Immune Cells
Neuropilin
N Mucosal Sia as Pathogen Decoys
Mucin
O Host Sias as Viral Traps
Sialylated Plasma Protein
P Sias as Biological Masks
Ashwell Receptor
Q Antibodies Against Sias
R Sias as "Xeno-autoantigens"
S Anti-Sia Antibodies Affect Reproductive Compatibility
T Sia based Blood Groups in Cats
Type A
Type B
U Modulation by Sia O-acetylation
V Alteration of IgG Fc Function by α2-6-sialylation

Figure 8: **Examples of roles of sialic acids [128]**. (A) Neu5Ac, the most common sialic acid in mammals (B) attribution of negative charge makes the cell hydrophilic (C) inhibition of complement fixation pathway (D) initiation of leukocyte extravasation. (E) engagement with siglecs causing inhibition or induction of cellular activation (F) pathogen binding (G) microbial interactions (H) modulation of immune cell function (I) alteration of host immune response by microbial mimicry of host Sias (J) fimbriae stabilization of microbes (K) Siglec-1 (sialoadhesin) expressed on macrophages recognizes pathogens expressing Sias and facilitates phagocytosis. (L) receptors for invasion of bacteriophages (M) regulate dendritic cell -T-cell interactions (N) decoys for Sia-binding microbes. (O) on erythrocytes and in plasma, can trap viruses (P) biological masks which inhibit constitutive activation of intrinsic receptors (Q) alter antigenicity of surface glycoconjugates (R) formation of “xeno-autoantigens,” (S) reproductive incompatibility during conception (T) blood-group determination in cats (U) O-acetylation of Sias modulate microbial lectin interactions (V) modulate effects of IgG antibodies (Adapted from [128])

2.12 Siglecs: Sialic acid binding immunoglobulin-like lectins

Siglecs are I-type lectins which recognize and interact with sialylated glycan residues on cell surfaces via immunoglobulin (Ig)-like domains. Structurally, siglecs are single-pass type I integral membrane proteins showing three different domains: extracellular N-terminal V-set Ig domain, followed by variable numbers of C2-set Ig domains, ranging from 16 in Sn to 1 in CD33, and a cytosolic domain having conserved tyrosine residues [129]. For sialic acid recognition by the siglecs, a conserved arginine residue is located in their V-set domains [130]. The intracellular domains have immuno-receptor tyrosine-based motifs in majority of the siglecs, which take part in signaling cascades. Taking into consideration the abundance of sialic acid residues, siglecs specifically bind either to sialylated glycans on the same cell surface, termed as *cis*-interaction, or binding to other cells, extracellular matrix protein or secreted glycoproteins, termed as *trans*-interactions. Due to their *cis*-interactions, the siglecs may be masked from making any *trans*-interactions. Sialidases are enzymes that cleave sialic acid residues, hence making them available for any trans-interactions, this phenomenon is called 'unmasking'. Siglecs can be divided into two gropus on the basis of evolutionary conservation and sequence similarity: an evolutionarily conserved subgroup (Siglecs-1, -2, -4 and -15) and a rapidly evolving CD33/Siglec- 3-related subgroup (Siglecs-3; -5–11 and -14, -16 in primates. Majority of the siglecs are expressed on the cells of the hematopoietic system, the exceptions being glial cell specific Siglec-4 (also known as myelin-

associated glycoprotein or MAG) and placental Siglec-6. The expression pattern of siglecs is very cell-type specific. The different cells of the immune system of both human and mouse express unique siglecs at different stages of development and activation, which is illustrated in Figure 9.

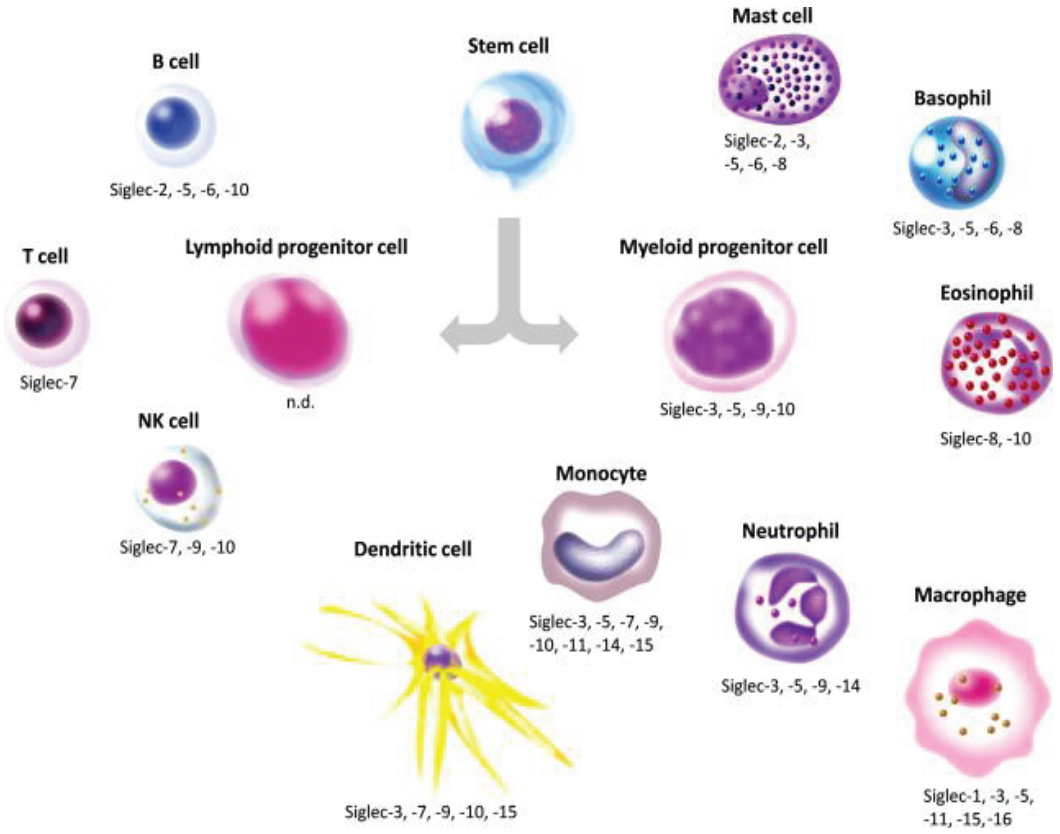

Figure 9. Siglec expression in hematopoietic and immune cells. Illustration shows the cell-type specific distribution of Siglecs in human hematopoietic and immune cells. [131]

Taking into consideration the fact that each siglec binds with highest affinity to a unique set of sialylated ligands, it has been assumed to carry out specific functions in the cell type that expresses it [132]. The intracellular domains of CD22 and most CD33-related siglecs have one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Normally, these ITIMs recruit tyrosine and inositol phosphatases and are known to act against signaling cascades involving other activatory receptors having immunoreceptor tyrosine-based activation motifs (ITAMs) These cellular signaling interactions play a role in the immune system [132]. The constitutive *cis*-interactions of the siglecs play a role in the suppression of immune responses by

modulating the activation states of these cells. On the other hand, the *trans*-interactions facilitate cell-cell cross talk and signaling. Thus both these *cis*- and *trans*-interactions may play an important role in immune system activation and triggering of inflammation.

The structures of the different siglecs expressed in human and mouse are illustrated in Figure 10.

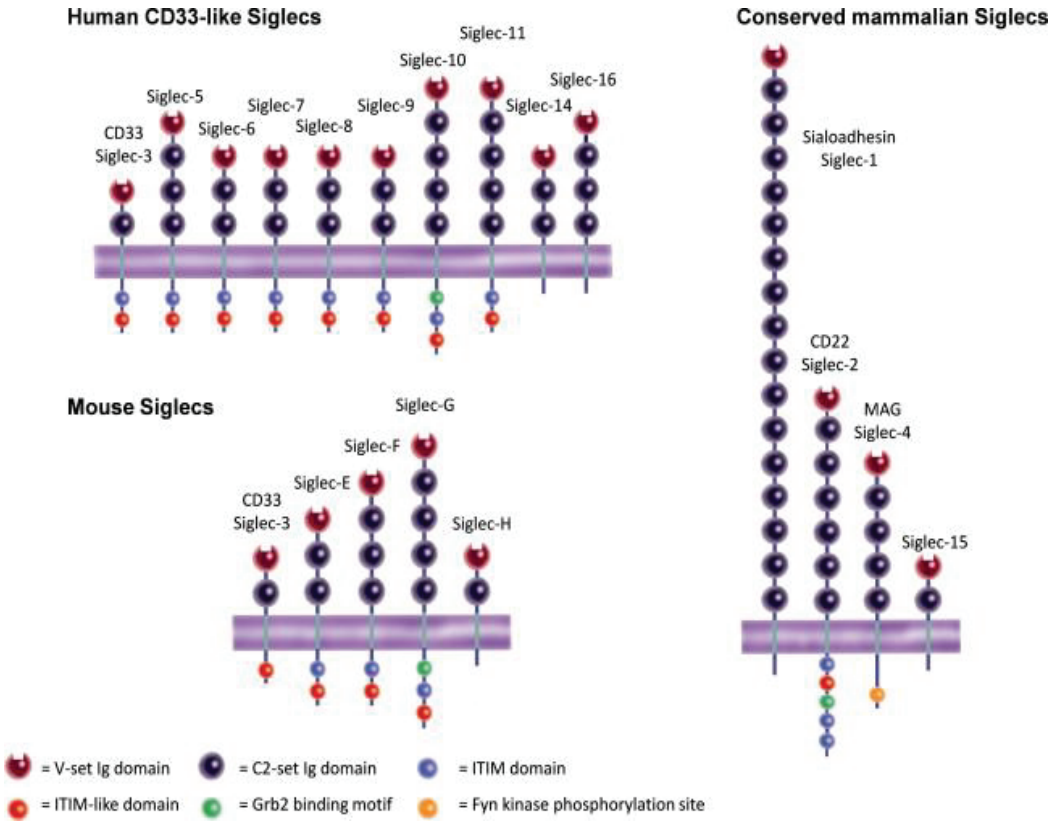

Figure 10: Nomenclature and structure of siglecs in humans and mice. [131]. Left: CD33-related siglec subfamily in human (top) and mice (bottom).,Right: evolutionary conserved siglec subfamily [131]

We hypothesized, that specific siglecs may be present in the pancreas due to the fact that inflammation contributes to the manifestation and progression of diabetes. After screening the human pancreatic tissue for siglecs expression, we found the expression of Siglec-3, -5 and -8 in the α-cells, Siglec-1, -2, -7 and -10 in the β-cell of the islets. Out of these siglecs, because of its observed regulation in diabetes, we focused our analysis on Siglec-7. Therefore, the characteristics of Siglec-7 are introduced in detail below.

2.12.1 Siglec-7: expression, structure and function

Siglec-7 is a CD-33 related siglec expressed at low levels on granulocytes, moderate levels on monocytes and dendritic cells, and relatively high levels on a major subset of NK-cells (CD56+) and a small subset of CD8+ T cells [133]. Its molecular structure consists of one NH 2-terminal V-type immunoglobulin-like domain which recognizes mainly α-2,8 sialic acid linkages, two C2-type, a trans-membrane region and a cytoplasmic tail containing two tyrosine residues located in immunoreceptor tyrosine-based inhibitory motifs [133, 134]. Functionally, Siglec-7 is an inhibitory receptor with regards to cell activation and differentiation. As seen in cord blood derived $CD34^+$ myeloid precursor cells, cross linking of Siglec-7 on these cells inhibited their proliferation and differentiation [135]. However, in contrast to the use of anti-Siglec-3 antibodies, cross linking of Siglec-7 on cells from several patients with acute myeloid leukemia exerted a strong inhibitory effect in distinct cases characterized by a high *in vitro* proliferation rate [136], suggesting that Siglec-7 impairs cell cycle progression and hence cells with a high proliferation rate are susceptible to a Siglec-7 mediated inhibition. In spite of the fact that Siglec-3 and Siglec-7 are expressed on the same cell and both recruit protein tyrosine phosphatase SHP-1, it remains to be elucidated by which mechanisms can they mediate different signaling events. Siglec-7 is an inhibitory receptor on human natural killer cells (NK cells) and monocytes [137]. Siglec-7 has been demonstrated to negatively regulate NK cell activation after engagement by antibodies [138] or binding with sialic acid-containing ligands [139]. Upon phosphorylation, it can majorly recruit the SH2 domain-bearing protein tyrosine phosphatase (PTP) SHP-1 [138]. Based on the presence of these siglecs on T cells, it was also shown that both Siglec-7 and Siglec-9 are capable of negative regulation of T cell receptor (TCR) signaling and that ligand binding is required for this activity [140], and ITIMs are critical for this inhibition mediated by Siglecs-7 and -9 [141]. Siglec-7 inhibits the FcRI-mediated serotonin release from RBL cells following cross-linking, in an ITIM dependant manner, recruiting SHP-1 and SHP-2 [141].

In summary, Siglec-7 can be considered as an inhibitory receptor, participating in the regulation of cellular activation, function and survival. We hypothesized that Siglec-7 expression in β-cells maintains cell survival and protects the cell from an overload of inflammatory signals as well as the migration of activated immune cells. Therefore, I investigated the role of Siglec-7 on β-cell survival and function in the context of

diabetes. In addition to this, Siglec-7 expression was also assessed on activated immune cells. Taken together, the present study focuses on the role of cell adhesion molecules, siglecs, in the pathophysiology of diabetes.

2.12.2 Involvement of siglecs in immune system responses

Since their discovery, siglecs have been shown to play varied roles in the immune system [142]. One of the main points that need to be considered in inferring the functions of these cell adhesion molecules is the fact that their ligands i.e. sialic acids are very ubiquitously expressed on every cell, as a result of which the adhesion molecules are interacting with 'self-ligands' as well. The constitutive masked state of the siglecs via endogenous *cis*-interactions plays a prominent role in the active state of the immune system [143]. Sialic acid recognition of the siglecs and the subsequent signaling is still poorly understood. To clarify the exact mechanisms behind the interaction dynamics, understanding of the mechanisms of masking and unmasking of the siglecs may shed light on the immunoregulatory events taking place in innate immunity [144, 145].

Focusing on Siglec-7, sialic acid recognition by Siglec-7 inhibited the cytoxicity of cells over-expressing GD3, a Siglec-7 ligand [139]. On similar lines, Siglec-7 and -9 over-expression in a human Jurkat T cell line inhibited T cell receptor signaling, which was lost after mutating the residues taking part in sialic acid binding [140]. Even though both Siglec-7 and -9 show high homology of structure and function, the extent to which they individually recruit the tyrosine phosphatases is different, the latter being stronger than the former [137]. Also the sialic acid residues that the recognize are different, endogenous ligand for Siglec-7 being GD3 and that of Siglec-9 being GD1a [146].

Taken together, these findings emphasize on the differential effects of sialic acid recognition of the siglecs, which play decisive roles in the activation of immune responses.

Alterations in the state of the immune system have been shown to be accompanied by alterations in siglec expression and function. Known triggers of immune system activation like LPS have been shown to induce Siglec-E (mouse orthologue) expression on bone marrow–derived macrophages in a TLR-dependant fashion [147]. This up-regulation is accompanied with suppression of the pro-inflammatory cytokine response that is a hallmark of such TLR engagement. Translating the results

to human Siglec-9, its inhibitory effect against TLRs was evidenced as a decrease in TNF-α and IL-6 production, which required its intact ITIM signaling [148]. In support of these findings, there is reduced expression of IL-12 after LPS stimulation of monocyte-derived dendritic cells observed after induction of Siglec-9 signaling in these cells [149]. Another important observation from these studies is that functional or structural orthologues of the human siglecs in mice do not always show identical regulation in these animals, as seen by the contradictory down-regulation of Siglec-9 in DCs exposed to LPS [149].

In terms of regulation of the inhibitory signaling of siglecs, not only is the extent of interaction between receptor and ligand crucial, but also modulation of cell-surface expression can be a mechanism of control. As is shown in a recent study, SH2-domain containing suppressor of cytokine signaling-3 (SOCS-3) competes with SHP1/2 for binding to human and Siglec-7 ITIMs [150] Ligation of SOCS-3 leads to recruitment of ECS E3 ligase which marks the Siglec-7 for proteosomal degradation. This in turn leads to loss of inhibition of cytokine-induced cell proliferation by Siglec-7, leading to regulation of inflammatory responses in these cells.

In summary, recent studies in this field support the hypothesis that siglecs are a family of receptors, which regulate the immune responses by affecting the proliferation, differentiation and activation of the cell-type expressing them. Thus, signaling cascades involving sialic acid recognition in a *trans* [144] or *cis* [140, 151] manner might take part in regulation of leukocyte responses in the event of inflammation.

2.11 Aims of the thesis

This thesis investigates different aspects of β-cell function and survival in the face of diabetes to explore 3 different angles to rescue the β-cell in diabetes, and answer 3 questions: (1) Are there any tools to induce proliferation in mature β-cell? (2) What are the effects DPP4 inhibitors on β-cell turnover and function? and (3) What are the players involved in the complex interplay of immune cells and β-cells within pancreatic islets? As introduced in the previous chapters, the phenomenon of β-cell loss is multi-factorial. Also, diabetes is a metabolic syndrome in which multiple organ systems are affected. Alongside with insulin resistance and chronic inflammation, T2D progresses after loss of β-cell function and mass. In this dissertation, I worked on diverse molecules which play a role in diabetes progression.

(1) In order to come up with strategies to rescue the β-cells from apoptosis, I investigated Fas/FasL mediated β-cell death, and introduced a novel regulator, TOSO, of this pathway in the β-cell. Whether TOSO is present and functioning in the pancreas has not been investigated, but would provide a promising tool to block Fas induced apoptosis also in β-cells. The advantage of TOSO would be to regulate endogenous FLIP levels to enhance the cell's proliferative capacity. Here, I provide evidence for constitutive expression of TOSO in the human β-cell and suggest a novel approach to prevent and treat diabetes by switching Fas signaling from apoptosis to proliferation.

(2) One of the aims of this thesis was to test whether a DPP-4 inhibitor would restore β-cell function and survival under diabetogenic conditions in human islets and whether cytokine production from islets under such conditions is affected. For our studies, Linagliptin, a xanthine-based, highly potent and long-acting DPP-4 inhibitor was used, which has recently been approved for the treatment of T2D (18, 19). This thesis provides evidence that Linagliptin improves β-cell survival and function in human islets through stabilization of GLP-1, which is secreted during islet culture.

(3) Taking into consideration the role of siglecs in an immune response and the implication of inflammation in the development of diabetes, I hypothesized that siglecs are expressed within pancreatic islets and are affected by a diabetic milieu and such changes in siglec expression could cause β-cell death and dysfunction. After screening human pancreas, analyses revealed differential expression of siglecs in the Islet of Langerhans. Specifically, Siglec-7 was expressed solely in the β-cell and was down-regulated in inflamed islets in diabetes. Restoring Siglec-7 expression in these islets could improve β-cell survival and function by inhibiting the pro-inflammatory responses. As Siglec-7 is known to be endogenously expressed in monocytes, I assessed it’s regulation during the activation of these cells observed in diabetes. As shown here, Siglec-7 expression is decreased in activated immune system. Finally, this thesis aimed to elucidate the significance of siglecs in the activation of immune cells, and brought forth the protective role of Siglec-7 in prevention of immune cell migration towards stressed and inflamed islets.

Thus, this thesis aimed to explore the β-cell destruction caused by chronic inflammation during the progression of diabetes. The ultimate goal is to bring forth novel players in the triggering of islet inflammation, which could be potential targets in prevention and/or cure of diabetes.

References

1. Gray, H., *Anatomy of the human body*. Philadelphia Lea & Febiger.
2. Bardeesy, N. and R.A. DePinho, *Pancreatic cancer biology and genetics.* Nat Rev Cancer, 2002. **2**(12): p. 897-909.
3. Youos, J.G., *The role of alpha-, delta- and F cells in insulin secretion and action.* Diabetes Res Clin Pract. **93 Suppl 1**: p. S25-6.
4. Hauge-Evans, A.C., et al., *Somatostatin secreted by islet delta-cells fulfills multiple roles as a paracrine regulator of islet function.* Diabetes, 2009. **58**(2): p. 403-11.
5. Batterham, R.L., et al., *Pancreatic polypeptide reduces appetite and food intake in humans.* J Clin Endocrinol Metab, 2003. **88**(8): p. 3989-92.
6. Asakawa, A., et al., *Characterization of the effects of pancreatic polypeptide in the regulation of energy balance.* Gastroenterology, 2003. **124**(5): p. 1325-36.
7. Joshi, S.R., R.M. Parikh, and A.K. Das, *Insulin--history, biochemistry, physiology and pharmacology.* J Assoc Physicians India, 2007. **55 Suppl**: p. 19-25.
8. Quesada, I., et al., *Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes.* J Endocrinol, 2008. **199**(1): p. 5-19.
9. Suckale, J. and M. Solimena, *Pancreas islets in metabolic signaling--focus on the beta-cell.* Front Biosci, 2008. **13**: p. 7156-71.
10. Hutton, J.C., *Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases.* Diabetologia, 1994. **37 Suppl 2**: p. S48-56.
11. WL Lowe, i.J.J., Totowa, NJ, Humana, *Principles of Molecular Medicine.* 1998.
12. Creutzfeldt, W., et al., *Inhibition of gastric secretion by fat and hypertonic glucose in the dog: role of gastric inhibitory peptide.* J Physiol, 1983. **334**: p. 91-101.
13. Drucker, D.J., et al., *Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line.* Proc Natl Acad Sci U S A, 1987. **84**(10): p. 3434-8.
14. Kreymann, B., et al., *Glucagon-like peptide-1 7-36: a physiological incretin in man.* Lancet, 1987. **2**(8571): p. 1300-4.
15. Gromada, J., J.J. Holst, and P. Rorsman, *Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1.* Pflugers Arch, 1998. **435**(5): p. 583-94.
16. Ishihara, H., et al., *Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells.* Nat Cell Biol, 2003. **5**(4): p. 330-5.
17. MacDonald, P.E., et al., *A K ATP channel-dependent pathway within alpha cells regulates glucagon release from both rodent and human islets of Langerhans.* PLoS Biol, 2007. **5**(6): p. e143.
18. Holst, J.J., *The physiology of glucagon-like peptide 1.* Physiol Rev, 2007. **87**(4): p. 1409-39.
19. Holst, J.J. and J. Gromada, *Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans.* Am J Physiol Endocrinol Metab, 2004. **287**(2): p. E199-206.
20. Rothenberg, M.E., et al., *Processing of mouse proglucagon by recombinant prohormone convertase 1 and immunopurified prohormone convertase 2 in vitro.* J Biol Chem, 1995. **270**(17): p. 10136-46.
21. Rouille, Y., et al., *Proglucagon is processed to glucagon by prohormone convertase PC2 in alpha TC1-6 cells.* Proc Natl Acad Sci U S A, 1994. **91**(8): p. 3242-6.
22. Rouille, Y., S. Martin, and D.F. Steiner, *Differential processing of proglucagon by the subtilisin-like prohormone convertases PC2 and PC3 to generate either glucagon or glucagon-like peptide.* J Biol Chem, 1995. **270**(44): p. 26488-96.
23. Rorsman, P., et al., *Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels.* Nature, 1989. **341**(6239): p. 233-6.
24. Cejvan, K., D.H. Coy, and S. Efendic, *Intra-islet somatostatin regulates glucagon release via type 2 somatostatin receptors in rats.* Diabetes, 2003. **52**(5): p. 1176-81.
25. Kurose, T., et al., *Glucagon, insulin and somatostatin secretion in response to sympathetic neural activation in streptozotocin-induced diabetic rats. A study with the isolated perfused rat pancreas in vitro.* Diabetologia, 1992. **35**(11): p. 1035-41.
26. Walker, J.N., et al., *Regulation of glucagon secretion by glucose: paracrine, intrinsic or both?* Diabetes Obes Metab, 2011. **13 Suppl 1**: p. 95-105.
27. Unger, R.H. and A.D. Cherrington, *Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover.* J Clin Invest, 2012. **122**(1): p. 4-12.
28. organisation, W.H., *Global status report on noncommunicable diseases 2010. Geneva*. 2010.
29. Kaul, K., et al., *Introduction to diabetes mellitus.* Adv Exp Med Biol. **771**: p. 1-11.
30. Harcourt, B.E., S.A. Penfold, and J.M. Forbes, *Coming full circle in diabetes mellitus: from complications to initiation.* Nat Rev Endocrinol. **9**(2): p. 113-23.

31. Organisation, W.H., *Fact sheet, general information.* 2013.
32. Tisch, R. and H. McDevitt, *Insulin-dependent diabetes mellitus.* Cell, 1996. **85**(3): p. 291-7.
33. Chao, C.C., et al., *The role of MHC class II molecules in susceptibility to type I diabetes: identification of peptide epitopes and characterization of the T cell repertoire.* Proc.Natl.Acad.Sci.U.S.A, 1999. **96**(16): p. 9299-9304.
34. Baisch, J.M., et al., *Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus.* N.Engl.J.Med., 1990. **322**(26): p. 1836-1841.
35. Mathis, D., L. Vence, and C. Benoist, *beta-Cell death during progression to diabetes.* Nature, 2001. **414**(6865): p. 792-798.
36. Bach, J.F. and D. Mathis, *The NOD mouse.* Res.Immunol., 1997. **148**(5): p. 285-286.
37. Wegmann, D.R., M. Norbury-Glaser, and D. Daniel, *Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice.* Eur J Immunol, 1994. **24**(8): p. 1853-7.
38. Yu, L., R. Gianani, and G.S. Eisenbarth, *Quantitation of glutamic acid decarboxylase autoantibody levels in prospectively evaluated relatives of patients with type I diabetes.* Diabetes, 1994. **43**(10): p. 1229-33.
39. Dotta, F., C. Fondelli, and U. Di Mario, *Type 1 diabetes mellitus as a polygenic multifactorial disease: immunopathogenic mechanisms of beta-cell destruction.* Acta Biomed, 2005. **76 Suppl 3**: p. 14-8.
40. Bertuzzi, F., et al., *Tissue factor and CCL2/monocyte chemoattractant protein-1 released by human islets affect islet engraftment in type 1 diabetic recipients.* J Clin Endocrinol Metab, 2004. **89**(11): p. 5724-8.
41. Lehuen, A., et al., *Immune cell crosstalk in type 1 diabetes.* Nat Rev Immunol, 2010. **10**(7): p. 501-13.
42. Florez, J.C., et al., *TCF7L2 polymorphisms and progression to diabetes in the Diabetes Prevention Program.* N Engl J Med, 2006. **355**(3): p. 241-50.
43. Weir, G.C. and S. Bonner-Weir, *Five stages of evolving beta-cell dysfunction during progression to diabetes.* Diabetes, 2004. **53 Suppl 3**: p. S16-21.
44. Jetton, T.L., et al., *Mechanisms of compensatory beta-cell growth in insulin-resistant rats: roles of Akt kinase.* Diabetes, 2005. **54**(8): p. 2294-304.
45. Steil, G.M., et al., *Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression.* Am J Physiol Endocrinol Metab, 2001. **280**(5): p. E788-96.
46. Chen, C., et al., *Mechanism of compensatory hyperinsulinemia in normoglycemic insulin-resistant spontaneously hypertensive rats. Augmented enzymatic activity of glucokinase in beta-cells.* J Clin Invest, 1994. **94**(1): p. 399-404.
47. Liu, Y.Q., T.L. Jetton, and J.L. Leahy, *beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats.* J Biol Chem, 2002. **277**(42): p. 39163-8.
48. Brunzell, J.D., et al., *Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests.* J Clin Endocrinol Metab, 1976. **42**(2): p. 222-9.
49. Dinneen, S., J. Gerich, and R. Rizza, *Carbohydrate metabolism in non-insulin-dependent diabetes mellitus.* N Engl J Med, 1992. **327**(10): p. 707-13.
50. de Luca, C. and J.M. Olefsky, *Inflammation and insulin resistance.* FEBS Lett, 2008. **582**(1): p. 97-105.
51. Samuel, V.T. and G.I. Shulman, *Mechanisms for insulin resistance: common threads and missing links.* Cell, 2012. **148**(5): p. 852-71.
52. Cai, D. and T. Liu, *Inflammatory cause of metabolic syndrome via brain stress and NF-kappaB.* Aging (Albany NY), 2012. **4**(2): p. 98-115.
53. Mathis, D., *Immunological goings-on in visceral adipose tissue.* Cell Metab, 2013. **17**(6): p. 851-9.
54. Butler, A.E., et al., *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-110.
55. Nauck, M., et al., *Reduced incretin effect in type 2 (non-insulin-dependent) diabetes.* Diabetologia, 1986. **29**(1): p. 46-52.
56. Meier, J.J. and M.A. Nauck, *Is the diminished incretin effect in type 2 diabetes just an epi-phenomenon of impaired beta-cell function?* Diabetes, 2010. **59**(5): p. 1117-25.
57. Woerle, H.J., et al., *The role of endogenous incretin secretion as amplifier of glucose-stimulated insulin secretion in healthy subjects and patients with type 2 diabetes.* Diabetes, 2012. **61**(9): p. 2349-58.
58. Bagger, J.I., et al., *Impaired regulation of the incretin effect in patients with type 2 diabetes.* J Clin Endocrinol Metab, 2011. **96**(3): p. 737-45.
59. Vilsboll, T., et al., *Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients.* Diabetes, 2001. **50**(3): p. 609-13.
60. Meier, J.J., et al., *Suppression of glucagon secretion is lower after oral glucose administration than during intravenous glucose administration in human subjects.* Diabetologia, 2007. **50**(4): p. 806-13.

61. Knop, F.K., et al., *Inappropriate suppression of glucagon during OGTT but not during isoglycaemic i.v. glucose infusion contributes to the reduced incretin effect in type 2 diabetes mellitus.* Diabetologia, 2007. **50**(4): p. 797-805.
62. Mentlein, R., B. Gallwitz, and W.E. Schmidt, *Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum.* Eur J Biochem, 1993. **214**(3): p. 829-35.
63. Lambeir, A.M., S. Scharpe, and I. De Meester, *DPP4 inhibitors for diabetes--what next?* Biochem Pharmacol, 2008. **76**(12): p. 1637-43.
64. Elashoff, M., et al., *Pancreatitis, pancreatic, and thyroid cancer with glucagon-like peptide-1-based therapies.* Gastroenterology, 2011. **141**(1): p. 150-6.
65. Foley, J.E., et al., *Beta cell function following 1 year vildagliptin or placebo treatment and after 12 week washout in drug-naive patients with type 2 diabetes and mild hyperglycaemia: a randomised controlled trial.* Diabetologia, 2011. **54**(8): p. 1985-91.
66. Brubaker, P.L. and D.J. Drucker, *Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system.* Endocrinology, 2004. **145**(6): p. 2653-9.
67. Li, L., et al., *Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B.* Diabetologia, 2005. **48**(7): p. 1339-49.
68. Stoffers, D.A., et al., *Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas.* Diabetes, 2000. **49**(5): p. 741-8.
69. Bulotta, A., et al., *Cultured pancreatic ductal cells undergo cell cycle re-distribution and beta-cell-like differentiation in response to glucagon-like peptide-1.* J Mol Endocrinol, 2002. **29**(3): p. 347-60.
70. Buteau, J., et al., *Glucagon-like peptide-1 prevents beta cell glucolipotoxicity.* Diabetologia, 2004. **47**(5): p. 806-15.
71. Cechin, S.R., et al., *Anti-Inflammatory Properties of Exenatide in Human Pancreatic Islets.* Cell Transplant, 2011.
72. Farilla, L., et al., *Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets.* Endocrinology, 2003. **144**(12): p. 5149-58.
73. Lupi, R., et al., *Effects of exendin-4 on islets from type 2 diabetes patients.* Diabetes Obes Metab, 2008. **10**(6): p. 515-9.
74. Drucker, D.J. and M.A. Nauck, *The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes.* Lancet, 2006. **368**(9548): p. 1696-705.
75. Pospisilik, J.A., et al., *Dipeptidyl peptidase IV inhibitor treatment stimulates beta-cell survival and islet neogenesis in streptozotocin-induced diabetic rats.* Diabetes, 2003. **52**(3): p. 741-50.
76. Cho, J.M., et al., *A novel dipeptidyl peptidase IV inhibitor DA-1229 ameliorates streptozotocin-induced diabetes by increasing beta-cell replication and neogenesis.* Diabetes Res Clin Pract, 2011. **91**(1): p. 72-9.
77. Pospisilik, J.A., et al., *Long-term treatment with dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulin sensitivity in the VDF Zucker rat: a euglycemic-hyperinsulinemic clamp study.* Diabetes, 2002. **51**(9): p. 2677-83.
78. May, M., *Novo awaits green light for diabetes drug.* Nat Biotechnol, 2009. **27**(8): p. 682-5.
79. Aston-Mourney, K., et al., *Too much of a good thing: why it is bad to stimulate the beta cell to secrete insulin.* Diabetologia, 2008. **51**(4): p. 540-5.
80. Wajchenberg, B.L., *beta-cell failure in diabetes and preservation by clinical treatment.* Endocr Rev, 2007. **28**(2): p. 187-218.
81. Gepts, W., *Pathologic anatomy of the pancreas in juvenile diabetes mellitus.* Diabetes, 1965. **14**(10): p. 619-33.
82. Eizirik, D.L. and T. Mandrup-Poulsen, *A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis.* Diabetologia, 2001. **44**(12): p. 2115-33.
83. Donath, M.Y., et al., *Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes.* J Mol Med, 2003. **81**(8): p. 455-70.
84. Moriwaki, M., et al., *Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus.* Diabetologia 1999 Nov;42(11):1332-40, (11): p. 40.
85. Maedler, K., et al., *Glucose induces beta-cell apoptosis via upregulation of the Fas-receptor in human islets.* Diabetes, 2001. **50**: p. 1683-1690.
86. Eizirik, D.L. and T. Mandrup-Poulsen, *A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis.* Diabetologia, 2001. **44**(12): p. 2115-2133.
87. Krammer, P.H., *CD95's deadly mission in the immune system.* Nature, 2000. **407**(6805): p. 789-795.
88. Loweth, A.C., et al., *Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation.* Diabetes, 1998. **47**(5): p. 727-732.

89. Aggarwal, B.B., et al., *Fas antigen signals proliferation of normal human diploid fibroblast and its mechanism is different from tumor necrosis factor receptor.* FEBS Lett, 1995. **364**(1): p. 5-8.
90. Irmler, M., et al., *Inhibition of death receptor signals by cellular FLIP.* Nature 1997 Jul 10;388(6638):190-5., (6638): p. 5.
91. Kataoka, T., et al., *The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways.* Curr Biol, 2000. **10**(11): p. 640-648.
92. Maedler, K., et al., *FLIP switches Fas-mediated glucose signaling in human pancreatic ß cells from apoptosis to cell replication.* Proc.Natl.Acad.Sci.U.S.A, 2002. **99**: p. 8236-8241.
93. Cottet, S., et al., *cFLIP Protein Prevents Tumor Necrosis Factor-alpha-Mediated Induction of Caspase-8-Dependent Apoptosis in Insulin-Secreting betaTc-Tet Cells.* Diabetes, 2002. **51**(6): p. 1805-1814.
94. Hitoshi, Y., et al., *Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells.* Immunity, 1998. **8**(4): p. 461-71.
95. Song, Y. and C.O. Jacob, *The mouse cell surface protein TOSO regulates Fas/Fas ligand-induced apoptosis through its binding to Fas-associated death domain.* J Biol Chem, 2005. **280**(10): p. 9618-26.
96. Huo, J., et al., *Genetic deletion of faim reveals its role in modulating c-FLIP expression during CD95-mediated apoptosis of lymphocytes and hepatocytes.* Cell Death Differ, 2009. **16**(7): p. 1062-70.
97. Scaffidi, C., et al., *The role of c-FLIP in modulation of CD95-induced apoptosis.* J Biol Chem, 1999. **274**(3): p. 1541-8.
98. Prentki, M. and C.J. Nolan, *Islet beta cell failure in type 2 diabetes.* J Clin Invest, 2006. **116**(7): p. 1802-12.
99. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease.* Nat Rev Immunol, 2011. **11**(2): p. 98-107.
100. Rhodes, C.J., *Type 2 diabetes-a matter of beta-cell life and death?* Science, 2005. **307**(5708): p. 380-4.
101. Donath, M.Y., et al., *Mechanisms of {beta}-Cell Death in Type 2 Diabetes.* Diabetes, 2005. **54 Suppl 2**: p. S108-13.
102. Maedler, K., *Beta cells in type 2 diabetes - a crucial contribution to pathogenesis.* Diabetes Obes Metab, 2008. **10**(5): p. 408-20.
103. Bensellam, M., D.R. Laybutt, and J.C. Jonas, *The molecular mechanisms of pancreatic beta-cell glucotoxicity: recent findings and future research directions.* Mol Cell Endocrinol, 2012. **364**(1-2): p. 1-27.
104. Donath, M.Y., et al., *Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes.* Diabetes, 1999. **48**(4): p. 738-744.
105. Pick, A., et al., *Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat.* Diabetes, 1998. **47**(3): p. 358-364.
106. Thomas, H.E., et al., *Beta cell apoptosis in diabetes.* Apoptosis, 2009. **14**(12): p. 1389-404.
107. Poitout, V., *Beta-cell lipotoxicity: burning fat into heat?* Endocrinology, 2004. **145**(8): p. 3563-5.
108. Robertson, R.P., et al., *Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes.* Diabetes, 2004. **53 Suppl 1**: p. S119-24.
109. Poitout, V., et al., *Gluco-lipotoxicity of the pancreatic beta cell.* Ann Endocrinol (Paris), 2004. **65**(1): p. 37-41.
110. Festa, A., et al., *Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study.* Diabetes, 2002. **51**(4): p. 1131-7.
111. Neels, J.G. and J.M. Olefsky, *Inflamed fat: what starts the fire?* J Clin Invest, 2006. **116**(1): p. 33-5.
112. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.
113. Maedler, K., et al., *Glucose-induced beta-cell production of interleukin-1beta contributes to glucotoxicity in human pancreatic islets.* J.Clin.Invest, 2002. **110**: p. 851-860.
114. Larsen, C.M., et al., *Interleukin-1-receptor antagonist in type 2 diabetes mellitus.* N Engl J Med, 2007. **356**(15): p. 1517-26.
115. Yuan, M., et al., *Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.* Science, 2001. **293**(5535): p. 1673-7.
116. Donath, M.Y., et al., *Islet inflammation in type 2 diabetes: from metabolic stress to therapy.* Diabetes Care, 2008. **31 Suppl 2**: p. S161-4.
117. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.
118. Homo-Delarche, F., et al., *Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat.* Diabetes, 2006. **55**(6): p. 1625-33.
119. Erber, W.N., *Human leucocyte differentiation antigens: review of the CD nomenclature.* Pathology, 1990. **22**(2): p. 61-9.

120. Schauer, R., *Sialic acids: fascinating sugars in higher animals and man.* Zoology (Jena), 2004. **107**(1): p. 49-64.
121. Kean, E.L., *Sialic acid activation.* Glycobiology, 1991. **1**(5): p. 441-7.
122. Shaper, J.H., et al., *Murine beta 1,4-galactosyltransferase. Analysis of a gene that serves both a housekeeping and a cell specific function.* Adv Exp Med Biol, 1995. **376**: p. 95-104.
123. Kelm, S. and R. Schauer, *Sialic acids in molecular and cellular interactions.* Int Rev Cytol, 1997. **175**: p. 137-240.
124. Varki, A., *Diversity in the sialic acids.* Glycobiology, 1992. **2**(1): p. 25-40.
125. Varki, A., *Sialic acids as ligands in recognition phenomena.* FASEB J, 1997. **11**(4): p. 248-55.
126. Collard, J.G., et al., *Cell surface sialic acid and the invasive and metastatic potential of T-cell hybridomas.* Cancer Res, 1986. **46**(7): p. 3521-7.
127. Saitoh, O., et al., *Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials.* J Biol Chem, 1992. **267**(8): p. 5700-11.
128. Varki, A. and P. Gagneux, *Multifarious roles of sialic acids in immunity.* Ann N Y Acad Sci, 2012. **1253**: p. 16-36.
129. Varki, A. and T. Angata, *Siglecs--the major subfamily of I-type lectins.* Glycobiology, 2006. **16**(1): p. 1R-27R.
130. Angata, T., N.M. Varki, and A. Varki, *A second uniquely human mutation affecting sialic acid biology.* J Biol Chem, 2001. **276**(43): p. 40282-7.
131. Jandus, C., H.U. Simon, and S. von Gunten, *Targeting siglecs--a novel pharmacological strategy for immuno- and glycotherapy.* Biochem Pharmacol, 2011. **82**(4): p. 323-32.
132. Crocker, P.R. and A. Varki, *Siglecs in the immune system.* Immunology, 2001. **103**(2): p. 137-45.
133. Nicoll, G., et al., *Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes.* J Biol Chem, 1999. **274**(48): p. 34089-95.
134. Angata, T. and A. Varki, *Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily.* Glycobiology, 2000. **10**(4): p. 431-8.
135. Vitale, C., et al., *Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15091-6.
136. Vitale, C., et al., *Surface expression and function of p75/AIRM-1 or CD33 in acute myeloid leukemias: engagement of CD33 induces apoptosis of leukemic cells.* Proc Natl Acad Sci U S A, 2001. **98**(10): p. 5764-9.
137. Yamaji, T., et al., *Characterization of inhibitory signaling motifs of the natural killer cell receptor Siglec-7: attenuated recruitment of phosphatases by the receptor is attributed to two amino acids in the motifs.* Glycobiology, 2005. **15**(7): p. 667-76.
138. Falco, M., et al., *Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells.* J Exp Med, 1999. **190**(6): p. 793-802.
139. Nicoll, G., et al., *Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms.* Eur J Immunol, 2003. **33**(6): p. 1642-8.
140. Ikehara, Y., S.K. Ikehara, and J.C. Paulson, *Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9.* J Biol Chem, 2004. **279**(41): p. 43117-25.
141. Avril, T., et al., *The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells.* J Immunol, 2004. **173**(11): p. 6841-9.
142. Crocker, P.R., J.C. Paulson, and A. Varki, *Siglecs and their roles in the immune system.* Nat Rev Immunol, 2007. **7**(4): p. 255-66.
143. Collins, B.E., et al., *Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6104-9.
144. Crocker, P.R. and A. Varki, *Siglecs, sialic acids and innate immunity.* Trends Immunol, 2001. **22**(6): p. 337-42.
145. Crocker, P.R., *Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling.* Curr Opin Struct Biol, 2002. **12**(5): p. 609-15.
146. Yamaji, T., et al., *A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9.* J Biol Chem, 2002. **277**(8): p. 6324-32.
147. Boyd, C.R., et al., *Siglec-E is up-regulated and phosphorylated following lipopolysaccharide stimulation in order to limit TLR-driven cytokine production.* J Immunol, 2009. **183**(12): p. 7703-9.
148. Ando, M., et al., *Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs.* Biochem Biophys Res Commun, 2008. **369**(3): p. 878-83.

149. Ohta, M., et al., *Immunomodulation of monocyte-derived dendritic cells through ligation of tumor-produced mucins to Siglec-9.* Biochem Biophys Res Commun, 2010. **402**(4): p. 663-9.
150. Orr, S.J., et al., *SOCS3 targets Siglec 7 for proteasomal degradation and blocks Siglec 7-mediated responses.* J Biol Chem, 2007. **282**(6): p. 3418-22.
151. Lajaunias, F., J.M. Dayer, and C. Chizzolini, *Constitutive repressor activity of CD33 on human monocytes requires sialic acid recognition and phosphoinositide 3-kinase-mediated intracellular signaling.* Eur J Immunol, 2005. **35**(1): p. 243-51.

TOSO promotes β-cell proliferation and protects from apoptosis

My contribution: Performed experiments of sequential thymidine analogue labelling, wrote the paper

Original article

MOLECULAR METABOLISM

TOSO promotes β-cell proliferation and protects from apoptosis

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ABSTRACT

Decreased β-cell mass reflects a shift from quiescence/proliferation into apoptosis, it plays a crucial role in the pathophysiology of diabetes. A major attempt to restore β-cell mass and normoglycemia is to improve β-cell survival. Here we show that switching off the Fas pathway using Fas apoptotic inhibitory protein (Faim/TOSO), which regulates apoptosis upstream of caspase 8, blocked β-cell apoptosis and increased proliferation in human islets. TOSO was clearly expressed in pancreatic β-cells and down-regulated in T2DM. TOSO expression correlated with β-cell turnover; at conditions of improved survival, TOSO was induced. In contrast, TOSO downregulation induced β-cell apoptosis. Although TOSO overexpression resulted in a 3-fold induction of proliferation, proliferating β-cells showed a very limited capacity to undergo multiple rounds of replication. Our data suggest that TOSO is an important regulator of β-cell turnover and switches β-cell apoptosis into proliferation.

Keywords TOSO; Fas; Islets; β-cell proliferation; Diabetes

INTRODUCTION

In both T1DM and T2DM, the major mechanism leading to decreased β-cell mass is increased β-cell apoptosis [1–3]. In T1DM, β-cell destruction occurs through immune mediated processes; mononuclear cell infiltration in the pancreatic islets and interaction between antigen presenting cells and T-cells lead to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other inflammatory products [4,5]. Cytokines and chemokines produced and secreted by activated macrophages, adipocytes and also by pancreatic β-cells have been suggested to initiate β-cell apoptosis [6]. Two major pathways trigger the onset of T1DM; the perforin/granzyme and the Fas/FasL system [7]. The Fas receptor (CD95), a 45-kDa type I transmembrane protein, is activated through interactions between antigen presenting β-cell and T-cells as well as through local expression of inflammatory mediators, i.e. cytokines, chemokines and other inflammatory compounds [5,7]. Fas binds to its ligand (FasL) and thus, initiates β-cell apoptosis [5].

FasL, a type II transmembrane protein of 40 kDa that can be secreted in a soluble form (26 kDa), is expressed primarily on activated T-lymphocytes [8] and also on β-cells [9,10]. Fas and Fas ligand are expressed in inflamed islets in pancreas sections of patients with recent-onset T1DM [11] as well as in patients with poorly controlled T2DM [9]. Triggering of Fas leads to the recruitment of Fas-associated death domain protein (FADD) and caspase-8 to the receptor (see Fig. 4). Binding of caspase-8 results in its activation by autoproteolytic cleavage and the release of the active subunits. The remaining caspase-8 prodomain is replaced by uncleaved procaspase-8, which then starts a new activation cycle.

A NOD mouse strain with a mutation in the Fas receptor, NOD *lpr/lpr*, did not develop diabetes [12]. β-cells from NOD mice that express a dominant-negative form of the Fas-associated death domain protein (FADD) were resistant to Fas induced cell death in vitro and in vivo. Furthermore, deletion of Fas protects islets from the toxic effects of cytokines [13] and of islet amyloid polypeptide (hIAPP) [14], which is suggested to be a major contributor to β-cell failure in T2DM [15].

In line with this observation, there are numerous studies on isolated rodent and human islets showing cytokine-induced Fas upregulation in the β-cell [10,16–18]. On the other hand, islets from NOD *lpr/lpr* were not protected against the autoimmune attack when transplanted into diabetic wild type recipients [19]; and only very few Fas-expressing β-cells were detected in islets of NOD mice at the onset of hyperglycemia [20]. Also, Fas signaling is needed for insulin secretion as shown in mice, pointing to a physiological role of the Fas receptor in β-cells. In human islets, an inhibitor of Fas-induced apoptosis, termed cellular FLICE (caspase-8)-inhibitory protein (FLIP) [21], was able to protect β-cells from cell death and restored β-cell function even under hyperglycemic conditions and in the presence of Fas. FLIP structurally resembles caspase-8 and thus interferes with its recruitment to the death-inducing signaling complex (DISC) and hence plays a critical role

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as an endogenous modulator of apoptosis [22]. Moreover, Fas signals do not always result in apoptosis but can also trigger a pathway that leads to proliferation [23]. Thereby, FLIP is pivotal in turning signals from cell death into those for cell survival/proliferation [24]. In β-cells, FLIP switched Fas activation from a death signal into a proliferation signal, and this may potentially expand β-cell mass [25]. The antagonistic anti-Fas antibody ZB4 inhibited the beneficial effect of FLIP at elevated glucose, demonstrating that Fas receptor activation is required for FLIP mediated proliferation. FLIP is also protective against cytokine-induced activation of caspase-8-dependent apoptosis [26].

A further upstream regulator of Fas is the cell surface protein TOSO, also named Fas apoptotic inhibitory protein (Faim3). It is expressed in activated T-cells [27,28]. TOSO negatively regulates FasL- and TNFα-induced apoptosis in lymphoma cell lines [29]. Also, a TOSO antibody potentiates TNFα induced apoptosis [29]. TOSO overexpressing Jurkat cells are resistant to Fas induced apoptosis through expression of FLIP [27]. FLIP expression levels are down-regulated in TOSO-deficient mice, causing these mice to be highly sensitive to Fas triggered apoptosis [30].

Thus, TOSO would provide a promising tool to block Fas induced apoptosis in β-cells, and its presence and function in human islets was investigated in the present study. The advantage of TOSO would be to regulate endogenous FLIP levels. These physiological FLIP levels are often not achieved by FLIP overexpression, and higher FLIP levels could even reverse its effect by induction of cell death. In the present study we provide evidence for constitutive expression of TOSO in the human β-cell and suggest a novel approach to prevent and treat diabetes by switching Fas signaling from apoptosis to proliferation. However, multiple rounds of self-duplication could not be achieved in human β-cells, confirming previous observations, which show that human β-cells have only a very limited capacity to self-duplicate [31].

MATERIAL AND METHODS

Islet culture

Human islets were isolated from pancreata of 8 healthy organ donors at the University of Lille or University of Chicago and cultured in CMRL-1066 medium as described previously [32]. Islets were cultured on extracellular matrix coated dishes derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel) for 4 days, allowing the cells to attach to the dishes and spread [33] and exposed to 5.5, 11.1, or 33.3 mM glucose or 5.5 mM plus recombinant human IL-1β (0.02–2 ng/ml, R&D Systems, Minneapolis, MN) or IFNγ (1000 U/ml, PeproTec, Rocky Hill, NJ, USA).

Transfection

At 2 days post-isolation and culture on extracellular matrix coated dishes, isolated islets were exposed to transfection using Ca^{2+}–KRH medium (KCl 4.74 mM, KH_2PO_4 1.19 mM, $MgCl_26H_2O$ 1.19 mM, NaCl 119 mM, $CaCl_2$ 2.54 mM, $NaHCO_3$ 25 mM, HEPES 10 mM). After 1 h incubation lipoplexes (Lipofectamine2000, Invitrogen, Carlsbad, CA, USA)/DNA ratio 2.5:1, 3 μg CMV-TOSO, RIP-TOSO, or CMV-GFP control plasmid DNA/100 islets or 50 nM siRNA to TOSO (RNAs of 21 nucleotides, designed to target human *TOSO*; ON-TARGETplus SMART-pool human FAIM3), (Dharmacon, Lafayette CO, USA) and scramble siRNA (Dharmacon) were added to transfect the cells as described previously [34,35]. After additional 6 h incubation, CMRL 1066 medium containing 20% FCS and L-Glutamine were added to the transfected islets. Transfection efficiency was determined using RT PCR, immunocytochemistry and western blotting. TOSO plasmid was obtained from the Full-Length Mammalian Gene Collection (invitrogen) and cloned into the pIRES2-AcGFP1 vector (invitrogen) named CMV-TOSO or into the pIT-HindIII-A-myc vector (kindly provided by T. Trüb, University Hospital Zurich), named RIP-TOSO.

Cell culture and thymidine analog administration

Post-transfection, the culture medium was supplemented with 10 μmol/L of CldU (Sigma, St. Louis, MO, USA, cat#C6891) for 1 or 2 days followed by 10 μmol/L of IdU (MP Biomedicals, Illkirch, France, cat#100357) for same time duration.

Glucose stimulated insulin secretion

Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated plates. For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose (stimulated). Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Islet insulin was determined using mouse insulin ELISA (ALPCO, Salem, NH, USA).

RNA extraction and RT-PCR analysis

Total RNA was isolated from cultured human islets as described previously [32]. For quantitative analysis, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with a commercial kit (Power SYBR Green PCR Master Mix; Applied Biosystems). Primers used: 5′CATGAACACAGA CCGGG3′/5′GAACTGGAGGGACCTTG-3′ (human TOSO), 5′GTTGGCCAGG CTGGTGTCCAG3′/5′CTGTGATGAGCTGCTCAGGGTGG3′ (human tubulin), and 5′TCACCCACACTGTGCCCATCTACGA3′/5′CAGCGGAACCGCTCATTGC CAATGG3′ (β-actin).

Western blot analysis

At the end of the incubation periods, islets were washed in ice-cold PBS and lysed for 40 min on ice in 40 μl lysis buffer containing 20 mM Tris acetate, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate. Prior to use, the lysis buffer was supplemented with Protease- and Phosphatase-inhibitors (Pierce, Rockford, IL, USA). Equivalent amounts of protein from each treatment group were run on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrically transferred onto PVDF membranes. Membranes were incubated with rabbit anti-TOSO and rabbit anti-$FLIP_L$ (C-19), (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and rabbit anti-β-actin (Cell Signaling Technology, Danvers, MA, USA) antibodies, followed by horseradish-peroxidase-linked anti-rabbit IgG.

Immunocytochemistry

Pancreatic sections from 8 healthy controls (4 lean and 4 obese) and from 7 patients (3 lean and 4 obese) with T2DM were obtained from the National Disease Research Interchange (NDRI), approval for the studies were granted by the Ethical Commission of Bremen University. For detection of β-cell TOSO expression insulin and TOSO staining were performed, 3 sections/pancreas were incubated in blocking buffer containing 0.2% Tween 20, 3% IgG-free Bovine serum albumin (BSA), 0.5% Triton X-100 for 1 h RT and overnight at 4 °C with rabbit anti-TOSO (Santa Cruz). Subsequently, all sections were double-stained for insulin and detected by donkey anti-guinea pig FITC-conjugated

antibody (Dako, Hamburg, Germany). To evaluate TOSO antibody specificity, the antibody was pre-incubated for 1 h at 37 °C with TOSO blocking peptide (Santa Cruz). Intensity and saturation of the staining was measured using Adobe Photoshop© Extended analysis software after an adapted model used by Pham et al. [36], expressed per islet area and normalized to the background signal.

For lineage tracing experiments, cultured islets were fixed in 4% paraformaldehyde for 30 min RT; permeabilized by 0.5% Triton-X-100 for 4 min RT and incubated in freshly diluted 1.5 N HCl for 40 min RT. After blocking for 1 h RT, staining for IdU and insulin was carried with by incubating with mouse anti-BrdU antisera (1:100) (Dako) and guinea pig anti-insulin (1:100) (Cat#18-0067; Zymed Laboratories Inc., San Francisco, CA, USA) overnight at 4 °C. The dishes were washed in a low salt TBST Buffer (36 mM Tris, 50 mM NaCl, 0.5% Tween-20; pH 8.0) and then in PBS. For CldU staining, dishes were incubated with Rat anti BrdU (Dako) (1:100; diluted in antibody dilution buffer) overnight at 4 °C. Incubation with secondary antibodies was carried out using AMCA donkey anti-Guinea Pig (1:100), Cy3 donkey anti-rat (1:100 and FITC donkey anti-mouse (1:100) at RT for 1 h).

For detection of β-cell apoptosis and proliferation, insulin and TUNEL or Ki67 staining (In Situ Cell Death Detection Kit, TMR red; Roche Diagnostics, Mannheim, Germany) were performed as described previously [37]. Fluorescent dishes were analyzed using Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon).

Statistical analysis

Samples were evaluated in a randomized manner by 2 investigators (G.D, S.L.) who were blinded to the treatment conditions. Data are presented as means ± SE and were analyzed by Student's *t*-tests. To account for multiplicity in the treated cells in vitro, a Bonferroni correction was used.

RESULTS

TOSO is down-regulated in diabetes and correlates positively with β-cell proliferation and negatively with β-cell apoptosis

To identify cell type specific TOSO localization in the human pancreas, we assessed TOSO expression in pancreatic sections from autopsy from lean and obese non-diabetic patients and from patients with T2DM. The specificity of the antibody was confirmed by pre-incubation with TOSO blocking peptide as a negative control (Fig. 1A, right panel). Constitutive expression of TOSO was detected in insulin producing β-cells in human pancreatic sections. TOSO was expressed in both lean and obese non-diabetic patients but depleted in lean and obese patients with T2DM (Fig. 1A). Quantification of the staining showed a 2.1-fold and 8.6-fold decrease in intensity and saturation in lean patients with T2DM vs. lean controls and a 3.5- and 9.9-fold decrease in intensity and saturation in obese patients with T2DM vs. obese controls, respectively (Fig. 1B).

To investigate whether such changes in TOSO expression occur during the process of the switch from proliferation into apoptosis, we analyzed TOSO expression in isolated human islets in response to short-term (12 h, Fig. 1C) and long-term (72 h, Fig. 1D) incubation with elevated glucose concentrations, 2 distinct conditions when glucose induced proliferation (Fig. 1F) or apoptosis (Fig. 1G).

TOSO was up-regulated dependent on glucose concentrations during 12 h of exposure; in contrast, TOSO was down regulated to almost undetectable levels after long-term incubation of the islets for 72 h (Fig. 1D), a condition where glucose induced β-cell apoptosis (Fig. 1G). Previously we have found that the cytokine IL-1β has also a dual physiological role on the regulation of β-cell function and survival [38,39], it induces proliferation at low and apoptosis at high concentrations (Fig. 1H). In line with these findings, low-dose IL-1β induced TOSO expression while high-dose almost depleted TOSO mRNA expression (Fig. 1E).

TOSO improves β-cell survival and function under diabetogenic conditions

To understand the physiological role of TOSO depletion in diabetes on the β-cell, the expression of TOSO was silenced in isolated human islets by using specific siRNAs. TOSO levels resulting from its overexpression and depletion were analyzed by immunocytochemistry (Fig. 2A), RT-PCR (Fig. 2D) and Western blotting (Fig. 2E). Protein was almost undetectable in islets upon siRNA treatment and RNA was 4.3-fold down-regulated (Fig. 2D), while overexpression was achieved in almost all islet cells even under elevated glucose or IL-1β/IFNγ exposure (Fig. 2A).

TOSO depletion resulted in a 3.3-fold induction of β-cell apoptosis, similar to cytokine exposure for 4 days (3.5-fold induction, Fig. 2A and B).

Also, β-cell apoptosis was 4-fold increased by the cytokine mix IL-1β/IFNγ and 3.5-fold by 33.3 mM glucose, which was prevented by CMV-TOSO overexpression.

On the other hand, TOSO overexpression induced a 2.6-fold increase in β-cell proliferation, as assessed by the mitotic marker Ki67 (Fig. 2C). β-cell proliferation decreased upon cytokine and elevated glucose treatment, which was back to basal levels upon TOSO overexpression. The pro-survival effect of TOSO expression was hypothesized to be due to the regulation of cFLIP, which leads to inhibition of apoptotic Fas signaling and acts as a switch to proliferation. Hence, we analyzed the levels of FLIP upon depletion and overexpression of TOSO. To address the effect of paracrine signaling, overexpression was carried out either specifically in the β-cells by using a rat insulin promoter driven expression plasmid (RIP-TOSO), or in all islet cell types using a cytomegalovirus driven plasmid (CMV-TOSO). Western blot analysis confirmed the efficient silencing and overexpression of TOSO at the protein level (Fig. 2E). Depletion of TOSO caused reduced FLIP expression; and, similarly, the increased amount of TOSO induced FLIP expression (Fig. 2E). β-cell proliferation was analyzed using the Ki67 antibody as shown in Fig. 2C, and was confirmed to be increased upon both β-cell specific and universal induction of TOSO expression, thus emphasizing its signaling in β-cells themselves (Fig. 2F).

The effect of the varying levels of TOSO expression on β-cell function was analyzed by carrying out glucose stimulated insulin secretion (GSIS) after depletion or overexpression of TOSO in presence and absence of diabetic stimuli. Elevated glucose and cytokines completely abolished GSIS, and also TOSO depletion resulted in a 2.2-fold ($p < 0.05$) reduction in the stimulatory index at basal conditions, but had no additive effect in islets exposed to diabetic milieu (Fig. 2G and H) at elevated glucose (33.3 mM) or the cytokine mixture. In contrast, TOSO overexpression improved GSIS 3.1-fold at elevated glucose levels and 1.4-fold ($p < 0.05$) at cytokine treatment (Fig. 2I and J), while at basal condition at 5.5 mM glucose, it had no effect.

TOSO overexpression induces early proliferation in β-cells

Sequential thymidine analog labeling is a recent and effective method for lineage tracing of proliferating cells, previously used in vivo to show the lack of any specialized β-cell progenitors in mice [40]. We

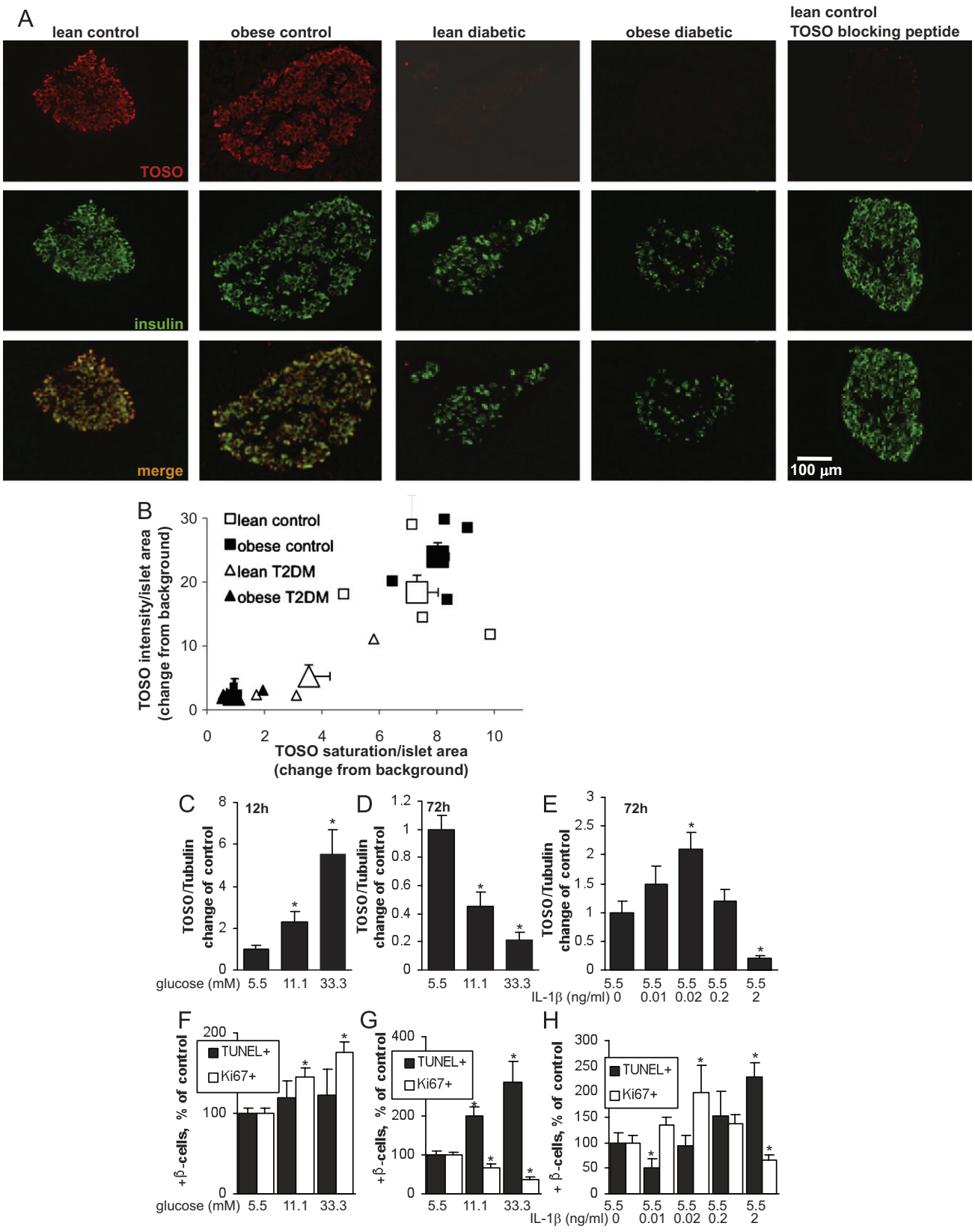

Fig. 1: TOSO is downregulated in T2DM and correlates positively with β-cell proliferation and negatively with β-cell apoptosis. (A,B) Representative co-staining for TOSO in red and insulin in green in human pancreatic sections from poorly controlled lean ($n=4$) and obese ($n=3$) patients with T2DM and lean ($n=4$) and obese ($n=4$) non-diabetic controls. From each pancreas, 3 sections were stained and analyzed and measurements for intensity and saturation are given as signal divided by islet area and normalized to background. Small symbols show means of all analyzed islets/pancreas, large symbols show means ± SE of all pancreases/group. (C–H) Human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to increasing concentrations of glucose (5.5–33.3 mM) for 12 (C,F) and 72 (D,G) h or to IL-1β (0.01–2 ng/ml) for 72 h (E,H). TOSO mRNA expression was analyzed by RT-PCR and expressed as relative changes of control and compared to Tubulin levels (C–E). In parallel, proliferation was measured by the Ki67 antibody stained in red and apoptosis analyzed by the TUNEL assay (F–H). Islets were triple-stained for insulin and counterstained for DAPI (not shown). Results are means ± SE of the percentage of Ki67- and TUNEL-positive β-cells. The average number of β-cells counted was 8150 for each treatment group in 3 separate experiments from 3 different organ donors.

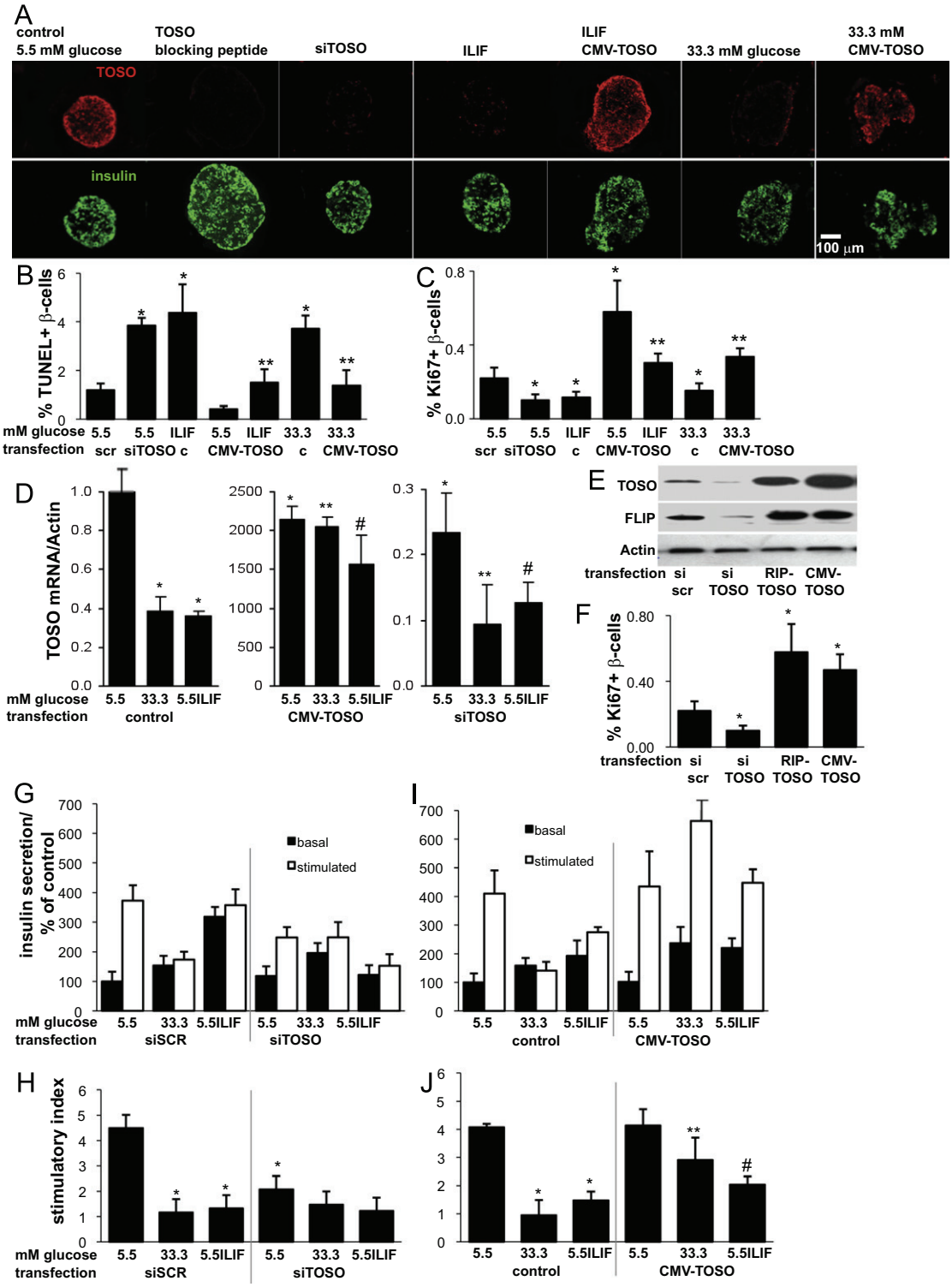

Fig. 2: TOSO improves β-cell survival and function under diabetogenic conditions. Human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to increasing concentrations of glucose (5.5–33.3 mM) or the cytokine mixture IL-1β (2 ng/ml) and IFNγ (1000 U/ml) for 72 h with or without depletion of TOSO by siTOSO or overexpression by CMV-TOSO transfection. TOSO was analyzed in each condition by immunostaining of Bouin-fixed paraffin-embedded islet pellets. As control, TOSO peptide was incubated with TOSO antibody before staining. Representative co-staining for TOSO in red and insulin in green are shown (A). Apoptosis was analyzed by the TUNEL assay in islet sections (B) and proliferation by the Ki67 antibody (C). Islets were triple-stained for insulin and counterstained for DAPI (not shown). Results are means ± SE of the percentage of Ki67- and TUNEL-positive β-cells. (D) TOSO mRNA expression was analyzed by RT-PCR and expressed as relative changes of control and compared to Tubulin levels. (E) Western blot analysis was performed 3 days after transfection with siRNA specific for TOSO, RIP-TOSO or CMV-TOSO plasmids. The same blot was analyzed for TOSO, c-FLIP (full length) and actin after stripping. (F) In parallel experiments, β-cell proliferation was assessed in islet pellets by double-staining for Ki67 and insulin. (B,C,F). The average number of β-cells counted was 7450 for each treatment group in 3 separate experiments from 3 different organ donors. (G–J) Glucose stimulated insulin secretion assays were performed after the 72 h culture period. (G,I) Basal (2.8 mM) and glucose stimulated (16.7 mM) insulin secretion was normalized to whole islet insulin content, respectively and expressed as percent change of basal conditions at 5.5 mM glucose. (H,J) Stimulatory index denotes the amount of glucose stimulated (16.7 mM glucose) divided by the amount of basal insulin secretion. Data are shown as mean ± SE from 3 islet isolations from 3 different donors. $p < 0.05$ to 5.5 mM glucose control,$^{**}p < 0.05$ to 33.3 mM glucose control, $^{\#}p < 0.05$ to IL/IF control.

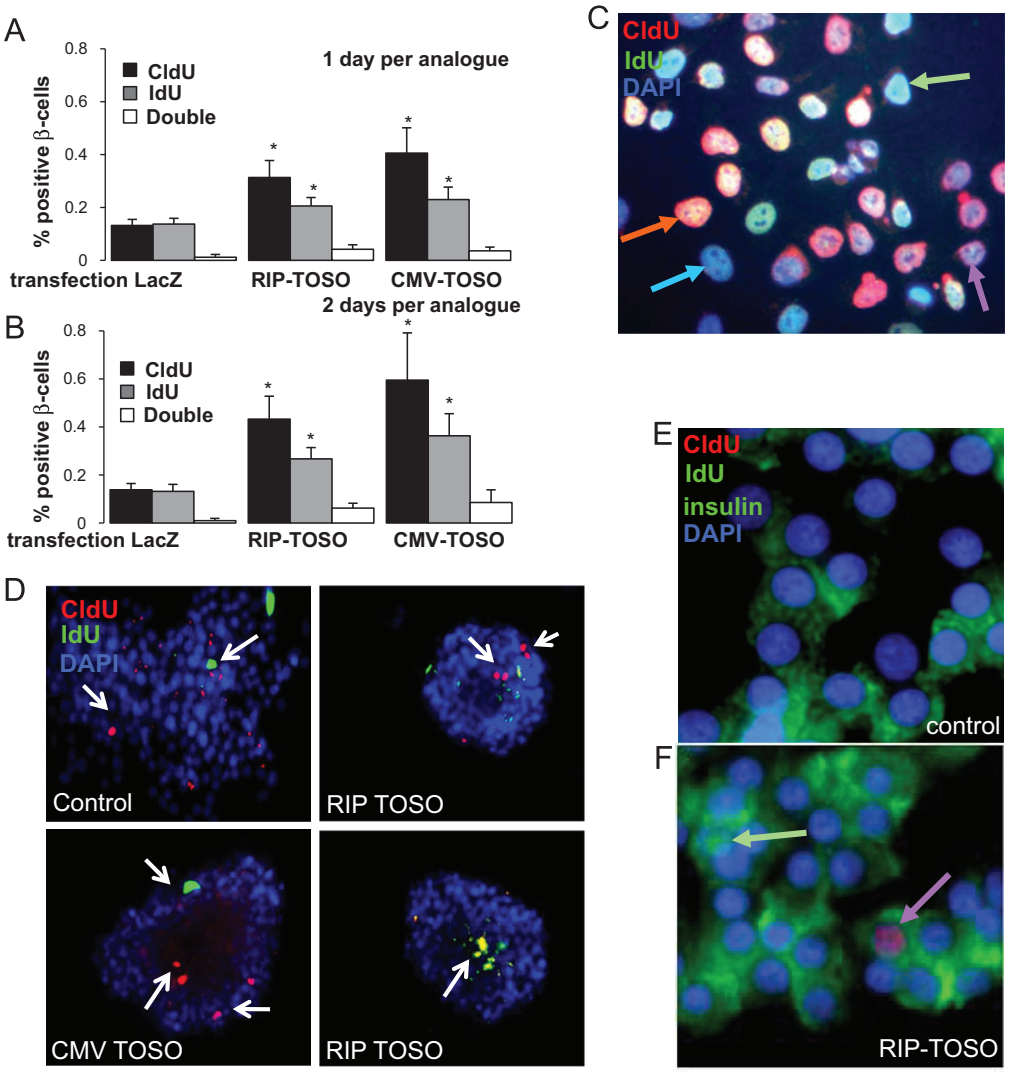

Fig. 3: TOSO over expression induces early proliferation in β-cells. Human pancreatic islets were cultured on extracellular matrix-coated dishes and TOSO overexpression was achieved by transfection with RIP-TOSO or CMV-TOSO. LacZ transfection was used as positive control. On day 1 (A) or day 2 (B) after the RIP/CMV-TOSO transfection, 10 μmol/L of the thymidine analog 5-chloro-2-deoxyuridine (CldU) and on day 2 (A) or day 4 (B) 5-iodo-2-deoxyuridine (IdU) were added into the islet culture medium. Thereafter, islets were fixed and labeled for thymidine incorporation by using specific antibodies against CldU and IdU (see material and methods). Results are means ± SE of the percentage of CldU/IdU-positive β-cells. The average number of β-cells counted was 7156 for each treatment group in 3 separate experiments from 3 different organ donors. (C,D) CldU/IdU and DAPI staining is shown in HeLa cells and human islets. CldU-positive cells are shown in red, IdU-positive cells are shown in green, and double positive cells are shown in orange (merged). Arrows show positive cells. In islets, only few double-positive cells were detected (D, panel d). (E,F) Triple staining for CldU in red, IdU in green (nucleus) and insulin in green (cytosol). Single-positive cells are shown (arrows). $^{*}p < 0.05$ to 5.5 mM glucose lacZ transfected control.

optimized the protocol to be used to detect multiple rounds of proliferation in vitro using immunocytochemistry.

To assess the nature of increased proliferation of β-cells upon TOSO overexpression, sequential thymidine analog labeling was carried out after overexpression of TOSO driven by the rat insulin promoter (RIP-TOSO) or cytomegalovirus promoter (CMV-TOSO). On day 1 and 2 after the RIP/CMV-TOSO transfection, we incorporated 10 μmol/L of the thymidine analogs 5-chloro-2-deoxyuridine (CldU) and 5-iodo-2-deoxyuridine (IdU), respectively for 1 day (Fig. 3A) into the islet culture medium. To exclude an effect of the expression changes of TOSO after overexpression and to take into account the number of days after transfection, analogs were also incorporated on day 2 and day 4 after transfection in a parallel experiment (Fig. 3B). The incorporation of the analogs, and thus the proliferation, was visualized using immunocytochemistry.

After quantification, it was observed that under control conditions, the extent of proliferation during administration of both the analogs was similar (Fig. 3A and B). In the TOSO transfected islets, there was an about 2-fold induction in β-cell proliferation (2.4-fold and 3.0-fold by RIP- and CMV-TOSO, respectively when CldU was administered over 1 day, Fig. 3A, and 3.1-fold and 4.3-fold by RIP- and CMV-TOSO, respectively when CldU was administered over 2 days, Fig. 3B). The percentage of β-cells undergoing proliferation during the administration of the second analog IdU was significantly less than that during the first CldU administration (1.5-fold and 1.7-fold decrease during IdU compared to CldU by RIP- and CMV-TOSO, respectively when analogs were administered over 1 day, Fig. 3A, and 2.0-fold and 2.8-fold increase during IdU administration by RIP- and CMV-TOSO, respectively when analogs were administered over 2 days, Fig. 3B). A very small percentage (0.04–0.08%) of co-labeled cells were observed in the TOSO transfected samples as compared to 0.01% in the control (Fig. 3A and B), suggesting a very limited capacity of β-cells to undergo multiple rounds of proliferation. To eliminate the possibility of preferential analog uptake, the sequence of analogs was reversed

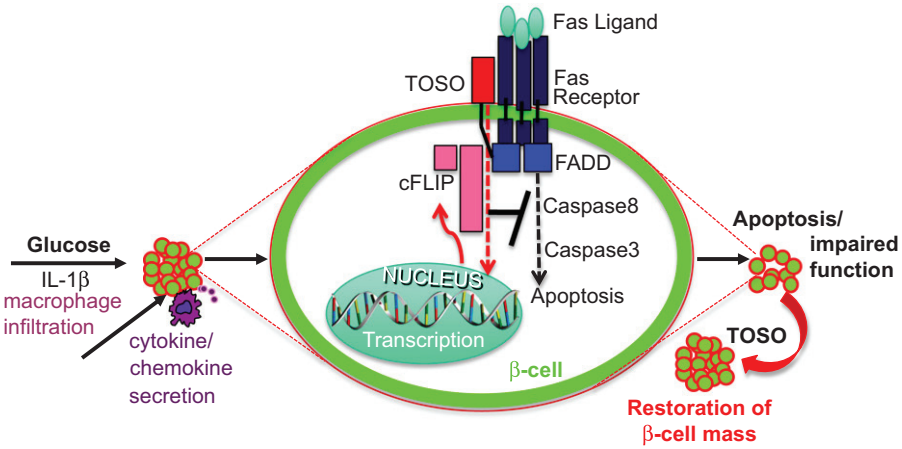

Fig. 4: Model of the role of TOSO in protecting the β-cell from Fas/FasL induced apoptosis. Elevated glucose levels and IL-1β, which is consequently secreted by β-cells or by infiltrating macrophages in diabetes, induce apoptosis and impair function through activation of the Fas receptor. Binding of the receptor to FasL, which is constitutively expressed by β-cells, leads to recruitment of the Fas associated death domain (FADD) to the receptor complex followed by cleavage of caspases and apoptosis. Direct binding of TOSO to FADD via its C-terminal tail at the cell membrane leads to increased levels of cFLIP which binds to FADD and inhibits further cleavage of Caspases. FLIP is also able to induce β-cell proliferation and thus may lead to the restoration of β-cell mass and function.

and the data obtained was found to be consistent (data not shown). Also, for taking into account the number of days after transfection, 2 different administration time durations were implemented, 2 days or 1 day per analog, which consistently showed that TOSO overexpression led to an induction of an early round of proliferation in the β-cells (Fig. 3A and B).

As a proof of principle, lineage tracing was carried out in HeLa cells and proliferating differentially labeled cells could be observed (Fig. 3C). Fig. 3D–F shows fluorescent microscopic images of a very limited number of cells inside the islets carrying the thymidine analog labels. Co-staining for insulin confirms the analysis in β-cells (Fig. 3E and F).

DISCUSSION

More than a decade ago, Hitoshi et al. cloned and characterized a novel immunoglobulin domain-containing type I transmembrane protein, TOSO, exhibiting potent pathway-specific anti-apoptotic effects in hematopoietic cells [27]. TOSO inhibited apoptosis induced by Fas-, TNFα-, FADD-, and PMA/ionomycin; but not staurosporine- or ceramide-induced apoptosis. The mechanism of inhibition and its specificity were hypothesized to be due to inhibition of caspase-8 processing through induction of cFLIP expression. The name TOSO was given after a Japanese liquor that is drunk on New Year's Day to celebrate long life and eternal youth [27], reflecting its pro-survival effect.

The role of TOSO in maintaining cFLIP expression during Fas mediated apoptosis of lymphocytes and hepatocytes has been elucidated using $TOSO^{-/-}$ mice [30]. B-cells and thymocytes from these mice show increased sensitivity to Fas-triggered apoptosis, and these mice suffer greater mortality and exhibit exacerbated liver damage in response to Fas engagement in vivo. TOSO modulates Fas-mediated apoptosis by influencing the expression of c-FLIP and regulating the physical binding of caspase-8 to Fas receptor.

The anti-apoptotic function of TOSO depends on ubiquitination of an adapter kinase, RIP1, and involves the recruitment of the death adapter FADD to a TOSO/RIP1 protein complex. Upon activation by FasL and TNFα, TOSO promotes the activation of pro-survival signaling pathways and protects from liver damage [29]. TLR activation leads to decreased TOSO expression shown in leukemic B-cells [41].

The activation of inflammatory pathways has been discovered as a causal event for β-cell destruction in diabetes [42,43], which is also mediated by TLR activation [32,44].

Thereby, not only pro-inflammatory compounds are activated (e.g. IL-1β, Fas), but also the anti-inflammatory cytokine interleukin-1 receptor antagonist and the anti-apoptotic cFLIP are down-regulated in β-cells in diabetes [9,42,45], and also TOSO downregulation was reported in response to TLR activation [41].

Thus, attempts to block Fas-induced apoptosis and the activation of pro-inflammatory cytokines could be a strategy to prevent diabetes. Here we show down-regulation of another protective factor in T2DM, and its loss promotes β-cell destruction.

TOSO expression correlated positively with β-cell proliferation; both the proliferation-inducing treatments i.e. acute high glucose and low dose IL-1β positively regulated TOSO expression. On the other hand, pro-apoptotic stimuli i.e. chronic elevated glucose and high dose IL-1β almost depleted TOSO mRNA expression. Thus, depletion of TOSO coincides with activation of β-cell apoptosis under conditions of stress. TOSO, when overexpressed, not only rescued β-cells from apoptosis but also triggered proliferation. This pro-survival signaling has also been observed in TOSO overexpressing Jurkat cells in which CD95L- and TNFα stimulation readily induced the activation of Erk1/2 [29]. While TNFα potentiates cell death, it is insufficient to cause apoptosis in β-cells [46]. Since TOSO promotes the TNFα induced signaling cascade, it is possible, that TNFα would induce β-cell apoptosis in the absence of TOSO, but there is no experimental proof for this hypothesis so far.

Intact Fas receptor signaling is also necessary for β-cell secretory function [47]. Hence, we investigated whether TOSO expression can also contribute to glucose stimulated insulin secretion. While TOSO overexpression was not effective at basal glucose levels, our results show that TOSO protected the inhibition of insulin secretion by elevated glucose and cytokines and restored β-cell function.

Mature human β-cells have only a very limited capacity to undergo proliferation [31]. To control and to foster β-cell proliferation has become a long-term goal in β-cell research. With the goal to find a subpopulation of β-cells with a higher proliferative capacity, we overexpressed TOSO and investigated the nature of the induced proliferation by using the technique of sequential thymidine analog labeling, previously developed in mice by Teta et al. [40].

We optimized the method so that it could be used in a similar fashion in vitro in human islets, to detect multiple rounds of proliferation. TOSO overexpression induced an early round of proliferation in the isolated human islets, irrespective of any effect of sequence of analog administration or time after transfection, however, only in a very limited number of islets (maximum 0.08%), more than one round of proliferation was observed during the 6-d culture period. Such limited proliferation capacity is in confirmation with the results from mouse β-cells in vivo [40,48].

CONCLUSIONS

In summary, TOSO is a novel anti-apoptotic protein, which interferes with the Fas triggered apoptosis by regulating FLIP and thereby initiating a pro-survival signaling cascade (see Fig. 4 for our model). Thus, TOSO hints to be a promising therapeutic target to rescue β-cells from apoptosis induced by elevated glucose and cytokines and, hence, intervening in the progression of diabetes. However, with the means of TOSO, we were unable to develop a β-cell, which would undergo multiple rounds of replication during culture.

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Conflict of interest.
None declared.

REFERENCES

[1] Kurrer, M.O., Pakala, S.V., Hanson, H.L., and Katz, J.D., 1997. Beta cell apoptosis in T cell-mediated autoimmune diabetes. Proceedings of the National Academy of Sciences of the United States of America 94:213–218.

[2] Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., et al., 2003. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110.

[3] Meier, J.J., Bhushan, A., Butler, A.E., Rizza, R.A., and Butler, P.C., 2005. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 48:2221–2228.

[4] Gepts, W., 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 14:619–633.

[5] Eizirik, D.L., and Mandrup-Poulsen, T., 2001. A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia 44:2115–2133.

[6] Donath, M.Y., Storling, J., Maedler, K., and Mandrup-Poulsen, T., 2003. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. Journal of Molecular Medicine 81:455–470.

[7] Mathis, D., Vence, L., and Benoist, C., 2001. beta-Cell death during progression to diabetes. Nature 414:792–798.

[8] Krammer, P.H., 2000. CD95's deadly mission in the immune system. Nature 407:789–795.

[9] Maedler, K., Spinas, G.A., Lehmann, R., Sergeev, P., Weber, M., et al., 2001. Glucose induces beta-cell apoptosis via upregulation of the Fas-receptor in human islets. Diabetes 50:1683–1690.

[10] Loweth, A.C., Williams, G.T., James, R.F., Scarpello, J.H., and Morgan, N.G., 1998. Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation. Diabetes 47:727–732.

[11] Moriwaki, M., Itoh, N., Miyagawa, J., Yamamoto, K., Imagawa, A., et al., 1999. Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. Diabetologia 42 (11), 1332–1340. [40].

[12] Chervonsky, A.V., Wang, Y., Wong, F.S., Visintin, I., Flavell, R.A., et al., 1997. The role of Fas in autoimmune diabetes. Cell 89:17–24.

[13] Allison, J., Thomas, H.E., Catterall, T., Kay, T.W., and Strasser, A., 2005. Transgenic expression of dominant-negative Fas-associated death domain protein in beta cells protects against Fas ligand-induced apoptosis and reduces spontaneous diabetes in nonobese diabetic mice. Journal of Immunology 175:293–301.

[14] Park, Y.J., Lee, S., Kieffer, T.J., Warnock, G.L., Safikhan, N., et al., 2012. Deletion of Fas protects islet beta cells from cytotoxic effects of human islet amyloid polypeptide. Diabetologia.

[15] Clark, A., Wells, C.A., Buley, I.D., Cruickshank, J.K., Vanhegan, R.I., et al., 1988. Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. Diabetes Research 9:151–159.

[16] Liu, D., Pavlovic, D., Chen, M.C., Flodstrom, M., Sandler, S., et al., 2000. Cytokines induce apoptosis in beta-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS−/−). Diabetes 49:1116–1122.

[17] Yamada, K., Takane-Gyotoku, N., Yuan, X., Ichikawa, F., Inada, C., et al., 1996. Mouse islet cell lysis mediated by interleukin-1-induced Fas. Diabetologia 39:1306–1312.

[18] Riachy, R., Vandewalle, B., Moerman, E., Belaich, S., Lukowiak, B., et al., 2006. 1,25-Dihydroxyvitamin D3 protects human pancreatic islets against cytokine-induced apoptosis via down-regulation of the Fas receptor. Apoptosis 11:151–159.

[19] Allison, J., and Strasser, A., 1998. Mechanisms of beta cell death in diabetes: a minor role for CD95. Proceedings of the National Academy of Sciences of the United States of America 95:13818–13822.

[20] Thomas, H.E., Darwiche, R., Corbett, J.A., and Kay, T.W., 1999. Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. Journal of Immunology 163:1562–1569.

[21] Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., et al., 1997. Inhibition of death receptor signals by cellular FLIP. Nature 388 (6638), 190–195. [Jul 10: 5].

[22] Tschopp, J., Irmler, M., and Thome, M., 1998. Inhibition of fas death signals by FLIPs. Current Opinion in Immunology 5:552–558. [8].

[23] Aggarwal, B.B., Singh, S., LaPushin, R., and Totpal, K., 1995. Fas antigen signals proliferation of normal human diploid fibroblast and its mechanism is different from tumor necrosis factor receptor. FEBS Letters 364:5–8.

[24] Kataoka, T., Budd, R.C., Holler, N., Thome, M., Martinon, F., et al., 2000. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. Current Biology 10:640–648.

[25] Maedler, K., Fontana, A., Ris, F., Sergeev, P., Toso, C., et al., 2002. FLIP switches Fas-mediated glucose signaling in human pancreatic β cells from

apoptosis to cell replication. Proceedings of the National Academy of Sciences of the United States of America 99:8236–8241.

[26] Cottet, S., Dupraz, P., Hamburger, F., Dolci, W., Jaquet, M., et al., 2002. cFLIP Protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. Diabetes 51:1805–1814.

[27] Hitoshi, Y., Lorens, J., Kitada, S.I., Fisher, J., LaBarge, M., et al., 1998. Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. Immunity 8:461–471.

[28] Song, Y., and Jacob, C.O., 2005. The mouse cell surface protein TOSO regulates Fas/Fas ligand-induced apoptosis through its binding to Fas-associated death domain. The Journal of Biological Chemistry 280:9618–9626.

[29] Nguyen, X.H., Lang, P.A., Lang, K.S., Adam, D., Fattakhova, G., et al., 2011. Toso regulates the balance between apoptotic and nonapoptotic death receptor signaling by facilitating RIP1 ubiquitination. Blood 118:598–608.

[30] Huo, J., Xu, S., Guo, K., Zeng, Q., and Lam, K.P., 2009. Genetic deletion of faim reveals its role in modulating c-FLIP expression during CD95-mediated apoptosis of lymphocytes and hepatocytes. Cell Death and Differentiation 16:1062–1070.

[31] Rutti, S., Sauter, N.S., Bouzakri, K., Prazak, R., Halban, P.A., et al., 2012. In vitro proliferation of adult human Beta-cells. PLoS One 7:e35801.

[32] Schulthess, F.T., Paroni, F., Sauter, N.S., Shu, L., Ribaux, P., et al., 2009. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. Cell Metabolism 9:125–139.

[33] Kaiser, N., Corcos, A.P., Sarel, I., and Cerasi, E., 1991. Monolayer culture of adult rat pancreatic islets on extracellular matrix: modulation of B-cell function by chronic exposure to high glucose. Endocrinology 129:2067–2076.

[34] Ardestani, A., Sauter, N.S., Paroni, F., Dharmadhikari, G., Cho, J.H., et al., 2011. Neutralizing IL-1{beta} induces {beta}-cell survival by maintaining PDX1 nuclear localization. Journal of Biological Chemistry.

[35] Le Bacquer, O., Shu, L., Marchand, M., Neve, B., Paroni, F., et al., 2011. TCF7L2 splice variants have distinct effects on {beta}-cell turnover and function. Human Molecular Genetics 20:1906–1915.

[36] Pham, N.A., Morrison, A., Schwock, J., Aviel-Ronen, S., Iakovlev, V., et al., 2007. Quantitative image analysis of immunohistochemical stains using a CMYK color model. Diagnostic Pathology 2:8.

[37] Shu, L., Sauter, N.S., Schulthess, F.T., Matveyenko, A.V., Oberholzer, J., et al., 2008. Transcription factor 7-like 2 regulates beta-cell survival and function in human pancreatic islets. Diabetes 57:645–653.

[38] Maedler, K., Schumann, D.M., Sauter, N., Ellingsgaard, H., Bosco, D., et al., 2006. Low concentration of interleukin-1{beta} induces FLICE-inhibitory protein-mediated {beta}-cell proliferation in human pancreatic islets. Diabetes 55:2713–2722.

[39] Meier, J.J., Ritzel, R.A., Maedler, K., Gurlo, T., and Butler, P.C., 2006. Increased vulnerability of newly forming beta cells to cytokine-induced cell death. Diabetologia 49:83–89.

[40] Teta, M., Rankin, M.M., Long, S.Y., Stein, G.M., and Kushner, J.A., 2007. Growth and regeneration of adult beta cells does not involve specialized progenitors. Developmental Cell 12:817–826.

[41] Vire, B., David, A., and Wiestner, A., 2011. TOSO, the Fcmicro receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation. Journal of Immunology 187:4040–4050.

[42] Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H.I., et al., 2002. Glucose-induced beta-cell production of interleukin-1beta contributes to glucotoxicity in human pancreatic islets. The Journal of Clinical Investigation 110:851–860.

[43] Donath, M.Y., Boni-Schnetzler, M., Ellingsgaard, H., Halban, P.A., and Ehses, J.A., 2010. Cytokine production by islets in health and diabetes: cellular origin, regulation and function. Trends in Endocrinology and Metabolism 21:261–267.

[44] Ehses, J.A., Meier, D.T., Wueest, S., Rytka, J., Boller, S., et al., 2010. Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. Diabetologia 53:1795–1806.

[45] Maedler, K., Sergeev, P., Ehses, J.A., Mathe, Z., Bosco, D., et al., 2004. Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. Proceedings of the National Academy of Sciences of the United States of America 101:8138–8143.

[46] Omori, K., Mitsuhashi, M., Ishiyama, K., Nair, I., Rawson, J., et al., 2011. mRNA of the pro-apoptotic gene BBC3 serves as a molecular marker for TNF-alpha-induced islet damage in humans. Diabetologia 54:2056–2066.

[47] Schumann, D.M., Maedler, K., Franklin, I., Konrad, D., Storling, J., et al., 2007. The Fas pathway is involved in pancreatic {beta} cell secretory function. Proceedings of the National Academy of Sciences of the United States of America 104:2861–2866.

[48] Teta, M., Long, S.Y., Wartschow, L.M., Rankin, M.M., and Kushner, J.A., 2005. Very slow turnover of beta-cells in aged adult mice. Diabetes 54:2557–2567.

The DPP-4 inhibitor linagliptin restores β-cell function & survival in human isolated islets through GLP-1 stabilization

My contribution: Performed islet culture, treatment, GSIS and RT-PCR experiments, wrote the paper

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The DPP-4 inhibitor linagliptin restores β-cell function & survival in human isolated islets through GLP-1 stabilization

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Context. **Inhibition of Dipeptidyl peptidase-4 (DPP-4) is a potent strategy to increase GIP and GLP-1 induced insulin secretion in diabetes. It is important to know if new drugs approved for the treatment of type 2 diabetes have direct effects on the β-cell.**

Objective. **Herein, we investigated the effect of linagliptin, a novel DPP-4 inhibitor, on β-cell function and survival.**

Design. **Human islets were exposed to a diabetic milieu (11.1–33.3 mM glucose, 0.5 mM palmitic acid, the mixture of 2 ng/ml IL-1β+1,000 U/ml IFN-γ or 50 μM H_2O_2) with or without 500 ng/ml Interleukin-1Receptor Antagonist (IL-1Ra) or 30–50 nM linagliptin.**

Results. **Linagliptin restored β-cell function and turnover, which was impaired when islets were exposed to elevated glucose, palmitate, cytokines or H_2O_2. Pre-treatment with IL-1Ra was similarly effective, except against H_2O_2 treatment. Nitrotyrosine concentrations in islet lysates, an indicator of oxidative stress, were highly elevated under diabetic conditions, but not in islets treated with linagliptin or IL-1Ra. Linagliptin also reduced cytokine secretion and stabilized GLP-1 in islet supernatants.**

Conclusions. **We show that the novel DPP-4 inhibitor linagliptin protected from gluco-, lipo- and cytokine-toxicity and stabilized active GLP-1 secreted from human islets. This provides a direct GLP-1 mediated protective effect of linagliptin on β-cell function and survival.**

Dipeptidyl peptidase 4 (DPP-4) is a ubiquitous cell-membrane protein enzyme responsible for cleaving and inactivating both incretins - glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (1), which are released by cells in the small intestine after the ingestion of food and which potentiate glucose stimulated insulin secretion (2). Patients with type 2 diabetes mellitus (T2D) have impaired insulin secretion, chronic hyperglycaemia and the effect of the incretins is significantly reduced (3).

Incretin-based therapies such as injectable GLP-1 receptor agonists or DPP-4 inhibitors are established treatments for T2D because of their glucose-dependent stimulation of insulin secretion, their inhibition of glucagon secretion and their intrinsic lack of risk for hypoglycemia (4), although an increased risk for pancreatitis has been discussed recently (5).

Long-term clinical effects of incretin-based drugs on the β-cell are still speculative (6). However, numerous in vivo and in vitro studies in rodents show that GLP-1 increases β-cell mass, proliferation (7, 8, 9) and β-cell neogenesis (10). GLP-1 inhibits β-cell apoptosis in human islets (11,

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Abbreviations:

12, 13), and a protective effect was also observed in islets isolated from patients with T2D (14).

Accordingly, stabilizing GLP-1 by DPP-4 inhibition restores glycemic control in diabetic animal models (2), improves β-cell survival, replication and neogenesis in the diabetic mouse and rat streptozotocin model (15, 16) and in the VDF diabetic rat (17).

The direct effect of DPP-4 inhibitors on human β-cells has not been investigated yet. Thus, the aim of this study was to test whether a DPP-4 inhibitor would restore β-cell function and survival under diabetogenic conditions in human islets and whether cytokine production from islets under such conditions is affected. For our studies, linagliptin, a xanthine-based, highly potent and long-acting-DPP-4 inhibitor was used, which has recently been approved for the treatment of T2D (18, 19). Interleukin-1 Receptor antagonist (IL-1Ra), which improves β-cell survival and function by neutralizing IL-1β (20, 21), was used as a positive control for a β-cell protective effect. In the present study we provide evidence that linagliptin improves β-cell survival and function in human islets through stabilization of GLP-1, which is secreted during islet culture.

Materials and Methods

Cell culture. Human islets were isolated from eight pancreata of healthy organ donors at Lille University and cultured in CMRL-1066 medium as described previously (22, 23). Islet purity was greater than 95% as judged by dithizone staining (if this degree of purity was not achieved by routine isolation, islets were handpicked). For long-term in vitro studies (96h), islets were cultured on extracellular matrix–coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), allowing the cells to attach to the dishes and spread, preserving their functional integrity (24). In parallel, islets were cultured in suspension dishes, treated for 72h, and fixed with Bouin´s solution. Islet sections were prepared as described previously (21).

Human islets were exposed to a diabetic milieu (11.1–33.3 mM glucose, or 5.5 mM glucose+0.5 mM palmitic acid, the mixture of 2 ng/ml recombinant human (rh) IL-1β (R&D Systems, Minneapolis, MN) +1,000 U/ml rh IFN-γ (PeProTech) for 72h or 50 μM H_2O_2 for 8h with or without 500 ng/ml Interleukin-1Receptor Antagonist (IL-1Ra), 10 nM-1 uM linagliptin, 200 nM sitagliptin, 100 nM Exendin- (9–39) (Bachem) or 0.5 nM GLP-1 (Sigma). Compounds were added 1h before the exposure to the diabetogenic conditions and throughout the culture. Palmitic acid was dissolved as described previously (25).

Ethical approval for the use of islets had been granted by the Ethical Committee of University of Bremen. We received the islets from the European Consortium For Islet Transplantation (ECIT). Whenever an islet isolation fails to be suitable for transplantation, centers provide them for islet research. Thus, these research projects comply with NIH regulations PHS 398, exemption 4. Human pancreata were harvested from brain dead donors, according to the European and National regulations for organ procurement. Donors or their family members gave written consent to donate organs for transplantation and research, all documented by the transplantation centers. Human islet isolations were performed with the approved protocols of the centers.

Cell turnover. For detection of β-cell apoptosis, 100 human islets were cultured in suspension dishes, treated for 72h, and fixed with Bouin´s solution. Islet sections were prepared as described previously, deparaffinized, rehydrated and incubated with 20 mg/ml proteinase K (Roche) for 15 min at 37°C before staining. In parallel, islets on ECM coated dishes were fixed using 4% paraformaldehyde and insulin costained with apoptosis analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, TMR red; Roche Applied Science) (26).

For analysis of β-cell proliferation, fixed islets on ECM coated dishes were incubated overnight at 4°C with mouse antihuman Ki67 (Invitrogen), followed by detection with donkey-rat Cy3-conjugated antibody (Jackson). Insulin was analyzed by anti guinea pig insulin antibody (Dako) followed by detection with donkey anti guinea pig FITC-conjugated antibody (Jackson). Islets and islet sections were embedded in Vectashield mounting medium. Fluorescence was analyzed using a *Nikon Eclipse Ti-U inverted research microscope* (Nikon GmbH, Dusseldorf, Germany) and images were acquired using NIS-Elements software (Nikon).

Glucose stimulated insulin secretion (GSIS). Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated dishes. For each independent experiment, 20 islets were plated and exposed to the treatment conditions as indicated above. For acute insulin release, islets were washed and preincubated (30 min) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose. The KRB was then replaced by KRB containing 2.8 mM glucose for 1 h (basal), followed by an additional 1 h incubation in KRB containing 16.7 mM glucose (stimulated). Islets were lysed in lysis buffer and whole islet protein measured by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL/Thermo Scientific). Insulin was determined using a human insulin ELISA kit (Alpco, Windham, NH) and normalized to whole islet cell lysate protein content.

Determination of nitrotyrosine. Nitrotyrosine concentration in human islets after the 4-d culture period was determined in islet cell lysates by an adapted ELISA method as previously described (27, 28). At the end of the incubation, islets (a total of 30 islets/condition) were washed in PBS, suspended, and lysed for 40 min on ice in lysis buffer (20 mM Tris acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate (all Sigma Aldrich) containing 1x protease and a phosphate inhibitor cocktail (Thermo Scientific, Rockford, IL USA). Protein concentrations of islet cell lysates were determined by BCA protein assay, and whole-cell lysates were stored at −80°C until assayed. The standard curve samples were prepared by dissolving 1 mg/ml BSA (Sigma) in 50 mmol/L KH_2PO_4. After addition of 1 mmol/L tetranitromethan (TNM), the dilution was incubated for 30 min at 37°C and adjusted to pH10 and the amount of nitrotyrosine present was measured at 430 nm and moles

nitrotyrosine for each mole protein was calculated directly using the Beer–Lambert law, $\epsilon = 4300\ M^{1}cm^{-1}$ (29). Standards of 0.05–0.6 pmol/μL were prepared. Maxisorp ELISA plate (BD Falcon#353279) was coated with standards and samples in coating buffer (50 mmol/l Na_2CO-$NaHCO_3$ buffer at pH 9.6) overnight at 4°C, washed 6 times with 0.05% Tween20 in PBS and incubated with IgG monoclonal antinitrotyrosin (Upstate Biotechnology, Lake Placid, NY/ Millipore) for 2h RT, washed and incubated for 45 min at 37°C with HPR conjugated goat anti-mouse (Zymed) and washed again. For the peroxidase reaction, 100μl tetramethyl-benzidine microwell peroxidase substrate (TBM; Sigma) was added for 10 min at RT and the reaction was stopped by adding 50 μl stop solution (BD Opty Kit B/ 0.5 M H_2SO_4) and read at 492 nm on a microplate reader. Concentration of nitrotyrosine was normalized to whole protein content of islet lysates.

Determination of cytokines, GLP-1 and DPP-4. After the 4-d culture period, human islet supernatants were measured for cytokine release using the Human ProInflammatory Tissue Culture Kit (*Meso Scale Discovery, MSD, Rockville, MD*). Total and active human GLP-1 was detected by the same assay platform (Meso Scale).

DPP-4 activity was detected using H-Ala-Pro-7-amido-4-trifluoromethylcoumarin (Ala-Pro-AFC; purchased from Bachem) in a 200 mM stock solution in dimethylformamide which was diluted 1000-fold with water before the assay. The assay itself was performed in black flat-bottom 96-well plates by mixing 50 μl of the diluted substrate (final concentration in the assay 100 μM), 25 μl of supernatant and 25 μl of assay buffer (100 mM Tris-HCl, 100 mM NaCl, pH 7.8). The plate was then incubated at room temperature for 1 h and fluorescence was measured at excitation/emission wavelengths of 405/535 nm. For standards, 20 μl of appropriate compound dilutions were prepared in assay buffer (compound stock solutions in dimethylsulfoxide (DMSO) final concentration in the assay 1%).

RNA extraction and RT-PCR analysis. Total RNA was isolated from cultured human islets by the Trizol method (peqGOLD TriFast, PeQLab, Erlangen, Germany). For quantitative analysis, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, CA, USA) with a commercial kit (Power SYBR Green PCR Master Mix or TaqMan(R) Fast Universal PCR Master Mix for TaqMan assays; Applied Biosystems). Primers used were 5′-ggctggtcatatggagggta-3′/5′-cagggctttggagatctgag-3′ (DPP-4), 5′-agagccaacgtcaagcatct-3′/5′-ctttagcttcgggtcaatgc-3′ (SDF1a), 5′-tccccaactcctgagttcac-3′/5′-acatcctcgacggcatctcag-3′ (IL-6), 5′tcctcctcggcttcagacac-3′/5′-gtaattggccgccacacagt (GLP-1R) 5′-gttggccaggctggtgtccag-3′/5′-ctgtgatgagctgctcagggtgg-3 (tubulin), 5′-ccaaccgcgagaagatga-3′/5′-ccagaggcgtacagggatag-3′ (actin) 5′tacgggtcctggcatcttgt3′/5′ccatttgtgttgggtccagc3′ (cyclophilin), 5′aaacggctaccacatccaag3′/ 5′cctccaatggatcctcgtta3′ (18S). TaqMan(R) Gene Expression Assays (Applied Biosystems) were used for IL-1β, IL-8, cyclophylin and tubulin.

Statistical analysis. Immunostainings were evaluated in a randomized manner by two investigators (P.S., S.L.) who were blinded to the treatment conditions Data are presented as means +/-SEM and were analyzed by paired Student's *t* test or by analysis of variance (ANOVA) with a Bonferroni correction for multiple group comparisons.

Results

Linagliptin, Sitagliptin and IL-1Ra improve β-cell survival

Loss of β-cells by apoptosis and decreased β-cell mass have been recognized as major pathological factors for the progression of diabetes. We tested the hypothesis whether linagliptin can protect β-cells from damage induced by diabetogenic conditions. Isolated human pancreatic islets were exposed for 4 d to increasing glucose concentrations (5.5, 11.1, 33.3 mM), 0.5 mM palmitate, the mixture of cytokines (2 ng/ml IL-1β+1,000 U/ml IFN-γ) or to 50 μM H_2O_2, for 8h, conditions which caused β-cell apoptosis and almost complete loss of proliferation. Islets were cocultured with 500 ng/ml IL-1Ra, which has been shown to improve β-cell survival (21) or with 50 nM linagliptin, which was added 1h before the diabetogenic conditions and maintained throughout the 4-d culture. At basal culture conditions of 5.5 mM glucose for 4 d, 10–1000 nM linagliptin had no influence on β-cell turnover (data not shown). Elevated glucose concentrations, free fatty acids, cytokines and oxidative stress induced β-cell apoptosis in isolated human islets, compared to control (2.5-, 3.6-, 3–1-, 3.7- and 4.5-fold induction by 11.1 and 33.3 mM glucose and by 0.5 mM palmitate, IL-1β+IFNγ and H_2O_2, respectively, $P < .001$, Figure 1A) in isolated human islets, compared to control incubations. This was reversed by coculture with IL-1Ra as well as with linagliptin. IL-1Ra reduced apoptosis 2.5, 2.0-, 2.1- and 3.0-fold in 11.1 mM and 33.3 mM glucose, palmitate and IL-1β+IFNγ, respectively ($P < .01$), but in H_2O_2, only a tendency but no significant protective effect was observed (Figure 1A). 50 nM linagliptin reduced apoptosis 2.3-, 3.8-, 2.0-, 2.5- and 2.1-fold in all diabetogenic conditions ($P < .01$, Figure 1A).

Human islets on the ECM-coated dishes had a very limited capacity to proliferate in culture (0.4 ± 0.1% Ki67/insulin double positive cells at 5.5 mM glucose basal condition). A diabetic milieu even further reduced this proliferative capacity (2.2-, 2.8-, 2.3-, 1.7- and 1.9-fold reduction by 11.1 and 33.3 mM glucose and by 0.5 mM palmitate, IL-1β+IFNγ when incubated for 4 d, and H_2O_2 when incubated for 8 h, respectively, compared to control, $P < .01$, Figure 1B). In contrast, IL-1Ra and linagliptin restored proliferation (4.3-, 5.8-, 4.0- and 3.1-fold induction by IL-1Ra and 4.8-, 6.8-, 4.4-, 2.1- and 4.1-fold by linagliptin in 11.1 and 33.3 mM glucose, in 0.5 mM palmitate, IL-1β+IFNγ and H_2O_2, respectively, compared to vehicle, $P < .01$, Figure 1B). IL-1Ra had no pro-

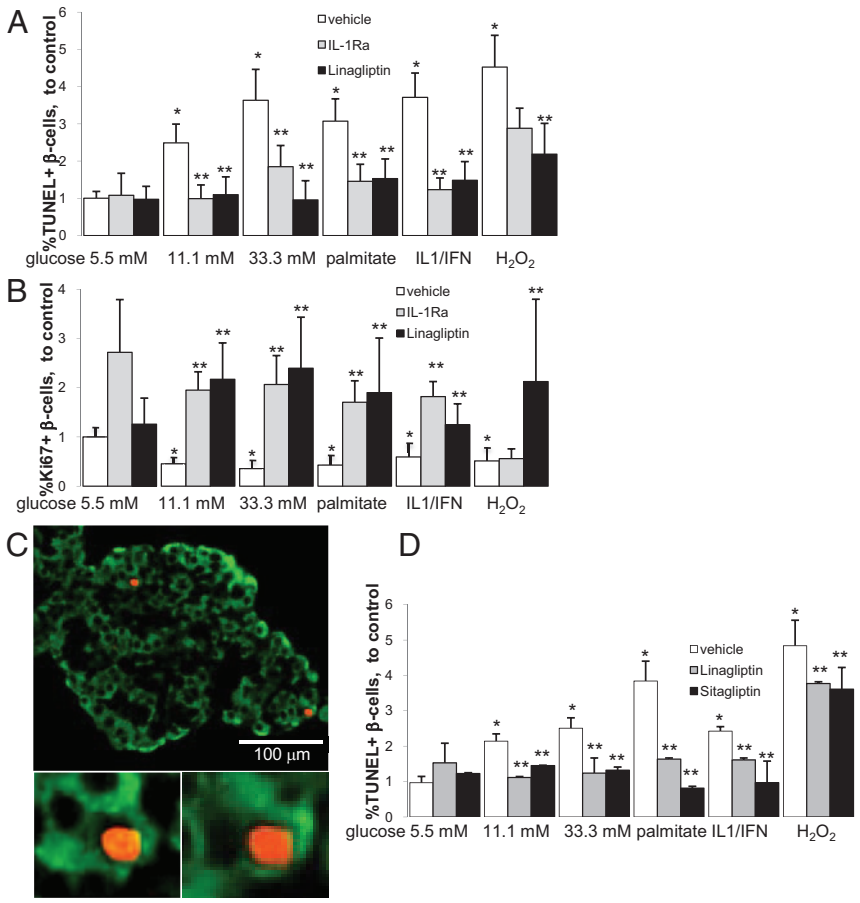

Figure 1. DPP-4 inhibition improves β-cell survival Human pancreatic islets were cultured on extracellular matrix coated dishes **(A,B)** or in suspension **(C,D)** at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate or 2 ng/ml IL-1β/1000 IU IFN-γ for 96h (**A,B**) or 72h (**C**,**D**) or at 50 μM H_2O_2 for 8h **(A-D)** in absence (control) or presence of IL-1Ra (500 ng/ml) or linagliptin (50 nM). Apoptosis was analyzed either directly in the dishes (**A**) or in paraffin embedded islet sections (**C,D**: red nuclei) by the TUNEL assay. Proliferation was analyzed in the dishes (**B**) by the Ki67 antibody. All specimens were double-stained in green for insulin. Results are means ±SEM of the TUNEL- or Ki67-positive β-cells, normalized to control conditions at 5.5 mM glucose (in absolute numbers: 0.4%TUNEL- and 0.37%Ki67-positive β-cells at 5.5 mM glucose on the extracellular matrix and 1.0% TUNEL-positive β-cells at 5.5 mM glucose in suspension culture). The average number of β-cells counted was 4175 for each treatment group from three different experiments from three different organ donors. ***C*** *shows a representative staining picture of a palmitate treated human islet (upper panel: x200 magnification) with 2 TUNEL-positive nuclei (power panel: x600 magnification).* $*P < .05$ compared to vehicle treated control at 5.5 mM glucose, $**P < .05$ compared to vehicle under the same treatment condition.

tective effect on β-cell proliferation under H_2O_2 exposure. This was also confirmed in mouse islets which were exposed for 8h to 50 μM H_2O_2 with or without 500 ng/ml IL-1Ra. Apoptosis was induced 3.6-fold by H_2O_2, while IL-1Ra failed to show any protective effect (data not shown).

To exclude a matrix-dependent effect, we cultured isolated human islets in suspension culture dishes, exposed them for 3 d or 8h (H_2O_2) to the above described diabetogenic milieu and analyzed β-cell apoptosis in bouin fixed paraffin embedded islet sections. A similar induction of β-cell apoptosis was observed by all diabetogenic culture conditions, linagliptin significantly reduced β-cell apoptosis (1.9-, 2.0-, 2.3-, 1.5 and 1.2-fold in 11.1 and 33.3 mM glucose, 0.5 mM palmitate, IL-1β+IFNγ and H_2O_2, compared to vehicle, $P < .01$; Figure 1D). Sitagliptin, another DPP-4 inhibitor tested in the same experiments had similar protective effects (Figure 1D; 1.4-, 1.9-, 4.7-, 2.5-, and 1.3-fold reduction of β-cell apoptosis in 11.1 and 33.3 mM glucose, 0.5 mM palmitate, IL-1β+IFNγ and H_2O_2, $P < .01$).

Linagliptin improves β-cell function and inhibits oxidative stress

In addition to its protective effect on β-cell survival, linagliptin improved glucose stimulated insulin secretion (GSIS) in islets after long-term culture of 4 d. In control conditions, there was a 4-fold stimulatory index when glucose was increased from 2.8 mM to 16.7 mM glucose (Figure 2D). All diabetogenic culture conditions significantly reduced GSIS ($P < .01$; Figure 2A,D). At basal glucose, neither IL-1Ra (Figure 2B,D) nor linagliptin (Figure 2C,D) increased the stimulatory index, but under the diabetogenic conditions GSIS was highly improved (1.2-, 1.4-, 1.9-, 2.3-, and 1.8- fold by IL-1Ra, Figure 2B,D and 1.9-, 2.5-, 2.1-, 2.4- and 2.5- fold by linagliptin, $P < .05$, Figure 2C,D).

To investigate the underlying mechanisms of the protective effect of the DPP-4 inhibitor, we hypothesized that changes in nitrotyrosine, a marker for oxidative stress, may occur during the long-term exposure with diabetogenic conditions. Nitrotyrosine production in islets during the 4-d culture period was measured and normalized to whole protein content of the islet lysate. Nitrotyrosine content was increased by glucose-, palmitate-, cytokine- and H_2O_2 exposure ($P < .05$; Figure 2E). Both IL-1Ra and linagliptin decreased nitrotyrosine under glucose, palmitate and cytokine treatment ($P < .05$), but not under conditions of H_2O_2 (Figure 2E).

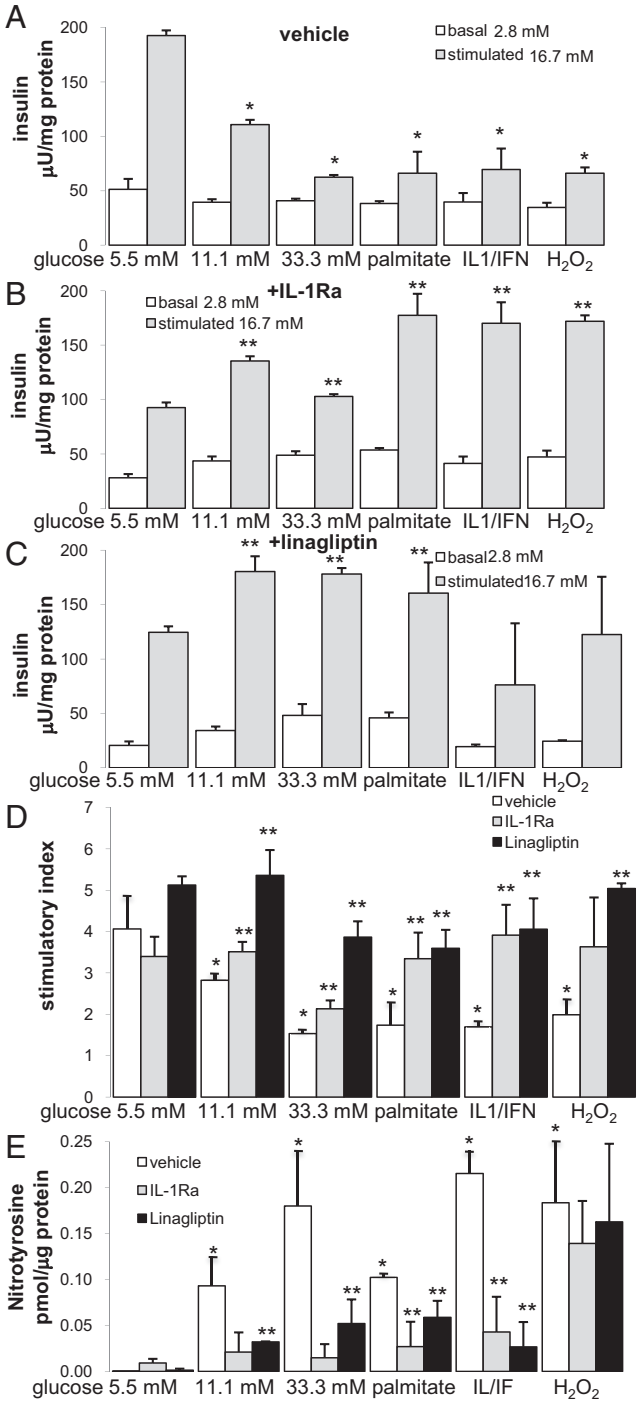

Figure 2. Linagliptin improves β-cell function and inhibits oxidative stress Human pancreatic islets were cultured on extracellular matrix coated dishes at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate or 2 ng/ml IL-1β/1000 IU IFN-γ for 96h or at 50 μM H_2O_2 for 8h in the absence (control; **A,D**) or presence of IL-1Ra (500 ng/ml; **B,D**) or linagliptin (50 nM; **C,D**). Basal and stimulated insulin secretion indicates the amount secreted during 1h incubations at 2.8 (basal) and 16.7 mM (stimulated) glucose following the culture period and normalized to protein content (**A-C**), stimulatory index was calculated as stimulated/basal insulin secretion **(D).** Nitrotyrosine content (**E**) in the islet lysates was normalized to whole islet protein content. Results are means ±SEM from triplicates from three independent experiments from three donors, $*P < .05$ compared to vehicle treated control at 5.5 mM glucose, $**P < .05$ compared to vehicle under the same treatment condition.

Linagliptin stabilizes GLP-1 in the supernatants of human islet cells

Since the main effect of DPP-4 is to stabilize GLP-1, we next investigated whether total and active GLP-1 levels in the culture medium were increased by linagliptin treatment. Diabetogenic conditions (increasing glucose concentrations and cytokines) induced accumulation of total GLP-1 (Figure 3A) in the islet supernatants, and linagliptin (30 nM) pretreatment resulted in approximately 2-fold higher total GLP-1 (Figure 3A) and approximately 3-fold higher active GLP-1 (Figure 3B) in all conditions. Levels of total and active GLP-1 were unchanged by IL-1Ra (data not shown).

Next, the effect of linagliptin on the stabilization of an exogenously added GLP-1 concentration was investigated. 1h after linagliptin (30 nM) treatment, 0.5 nM GLP-1 (a concentration similar to the secreted total GLP-1 from islets treated with Linagliptin) was added to the islet culture medium and immediately measured. Rapid degradation of GLP-1 was observed during culture (Figure 3C,D), while addition of linagliptin inhibited such degradation of GLP-1. Total (Figure 3C) and active GLP-1 (Figure 3D) accumulated during culture in the linagliptin treated islet supernatants, which did not occur in the non-treated islets.

GLP-1 stabilization was accompanied by restored GLP-1 receptor levels. In confirmation with earlier studies (30, 31), GLP-1 receptor levels were decreased in response to glucotoxicity and cytokine exposure, compared to untreated control. In contrast, 30 nM linagliptin increased GLP-1 receptor levels in all diabetogenic conditions (Figure 3E). To further prove whether the effect of linagliptin is GLP-1 dependent, we cocultured linagliptin with Exendin- (9–39), an established inverse agonist of the GLP-1 receptor (32, 33). While 30 nM linagliptin was effective in improving glucose stimulated insulin secretion under cytokine exposure, the addition of 100 nM Exendin- (9–39) inhibited the linagliptin effect (Figure 3F). To prove that linagliptin was active, we also measured DPP-4 activity in the islet cell supernatants. As expected, linagliptin inhibited DPP-4 activity in culture (Figure 3G). The potential GLP-1-SDF-1α synergism strongly promotes β-cell survival (34). Since SDF-1α is another substrate of DPP-4 (35) and an important prosurvival factor for β-cells (36), we tested whether increases in GLP-1 concentration and reduction in DPP-4 activity by linagliptin are also paralleled with SDF1α mRNA production. Linagliptin strongly increased SDF1α under basal as well as under diabetogenic conditions ($P < .05$, Figure 3H), while glucose and palmitate themselves or IL-1Ra treatment of the islets had no effect on SDF-1α (Figure 3H).

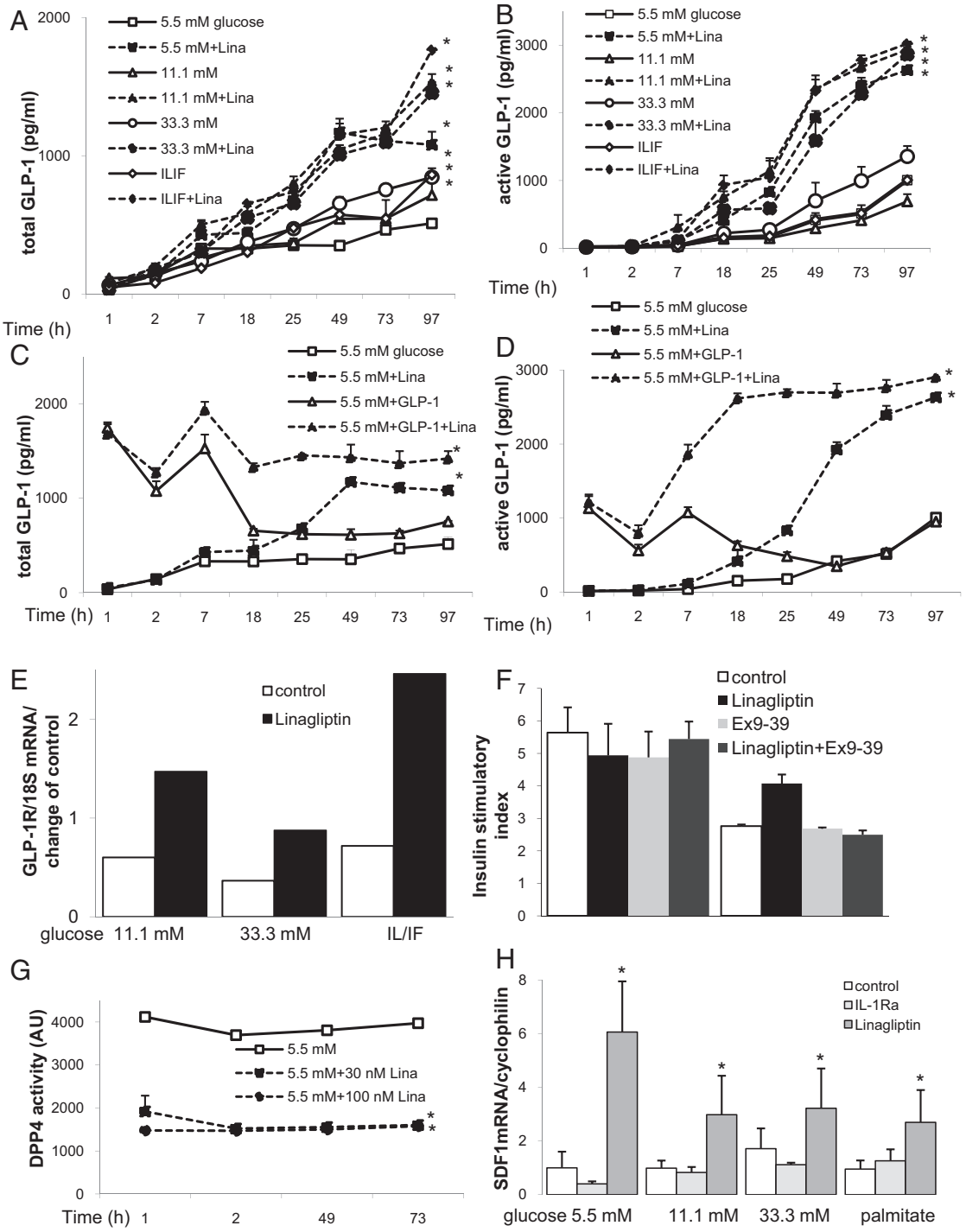

Figure 3. Linagliptin stabilizes GLP-1 in the supernatants of human islet cells Human pancreatic islets were cultured on extracellular matrix coated dishes at 5.5 mM glucose (control) or 11.1 and 33.3 mM glucose, 0.5 mM palmitate or 2 ng/ml IL-1β/1000 IU IFN-γ for 96h in the absence (control) or presence of linagliptin (50 nM, **A,B)** or 0.5 nM GLP-1 **(C,D)**. Total (**A,C**) and active (**B,D**) GLP-1 secretion was measured in the supernatants during the 4-d culture period. mRNA was isolated from treated islets after 4 d and RT-PCR analysis of GLP-1 receptor levels (**E**) was performed. The levels of gene expression were normalized to tubulin and 18S and presented as change of 5.5 mM glucose control. Human pancreatic islets were cultured at 5.5 mM (control) glucose or 2 ng/ml IL-1β/1000 IU IFN-γ for 96h in presence of 30 nM linagliptin and an inverse agonist of the GLP-1 receptor Exendin- (9–39) (100 nM, **F**). Insulin stimulatory index as the ratio of the stimulated and basal insulin secretion was calculated. (**G**) DPP-4 activity was measured at control conditions and with 30 and 100 nM linagliptin during culture. **(H)** mRNA was isolated from treated islets after 4 d of culture and RT-PCR analysis of SDF-1α performed. The levels of gene expression were normalized to tubulin and cyclophilin (with similar results) and presented as change of control. Results are means ±SEM from triplicates from three independent experiments from three donors, except for (**E**): data are from one single donor (in triplicates) and (**F**): from 2 donors (each in triplicates), $*P < .05$ compared to vehicle treated control at 5.5 mM glucose.

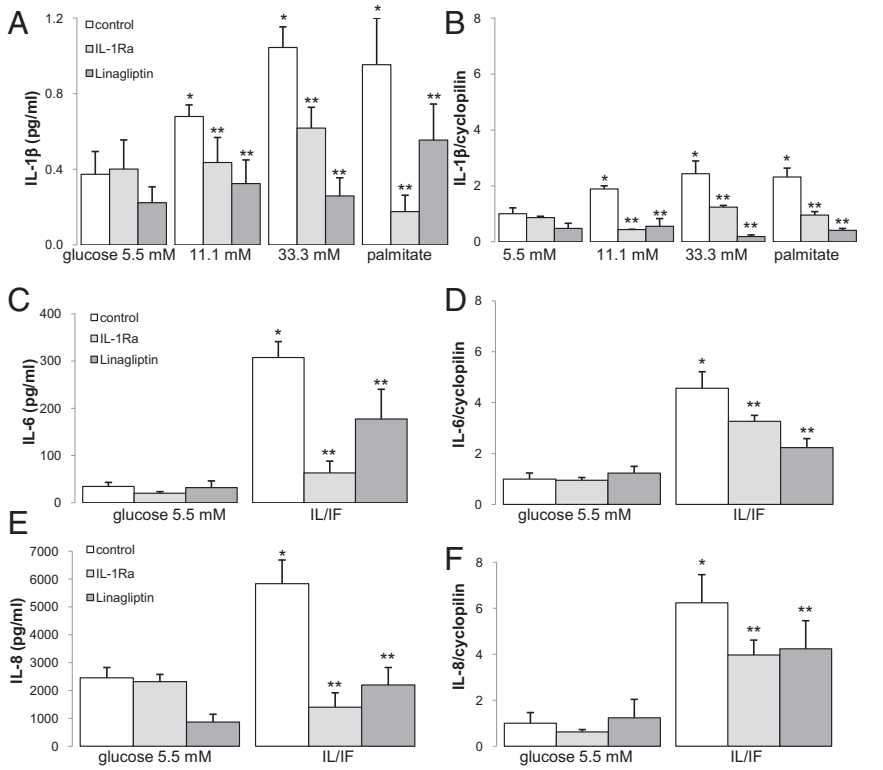

Figure 4. Linagliptin inhibits cytokine secretion from islets under diabetogenic conditions Human pancreatic islets were cultured on extracellular matrix coated dishes at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate or 2 ng/ml IL-1β/1000 IU IFN-γ for 96h in the absence (control) or presence of IL-1Ra (500 ng/ml) or linagliptin (50 nM). **(B,D,F)** mRNA was isolated from treated islets after 4 d and RT-PCR analyses of IL-1β (**B**), IL-6 (**D**) and IL-8 (**F**) were performed. The levels of gene expression were normalized to tubulin and cyclophilin (with similar results) and presented as change of control. Cytokine and chemokine secretion of IL-1β (**A**), IL-6 (**C**), IL-8 (**E**) from the culture medium of the same islets were analyzed in a multiplex assay (Meso Scale Discovery©). Results are means ±SEM from three independent experiments from three donors, $*P < .05$ compared to vehicle treated control at 5.5 mM glucose, $**P < .05$ compared to vehicle under the same treatment condition.

Linagliptin inhibits cytokine secretion and production from islets under diabetogenic conditions

Since proinflammatory cytokines and chemokines are elevated under a diabetogenic milieu, we tested whether IL-1Ra and linagliptin exert their protective effects through an inhibition of cytokine secretion. Cytokine and chemokine secretion into the culture medium was measured simultaneously by the MSD© technique and mRNA production by RT-PCR. The diabetogenic milieu which included elevated glucose concentrations and palmitate significantly increased IL-1β secretion (Figure 4A) and mRNA production (Figure 4B), while IL-6 (Figure 4C,D) and IL-8 (Figure 4E,F) were increased by the cytokine mixture IL-1β and IFNγ, but no significant effect was observed in the other diabetogenic conditions. While IL-1Ra and linagliptin had no effect on the minimal basal IL-1β secretion and production during the 4-d culture period, they significantly decreased both IL-1β secretion and mRNA production (Figure 4A,B) under diabetogenic conditions. Similarly, IL-6 and IL-8, which were elevated in response to IL-1β/IFNγ were decreased by IL-1Ra and linagliptin (Figure 4C-F).

Discussion

Orally administered DPP-4 inhibitors prevent the rapid cleavage of GLP-1, and thus increase levels of active GLP-1, resulting in increased insulin and reduced glucagon secretion, lowered glucose and reduced HbA1c levels by 0.5–1.0% (2). Linagliptin improves glycemic control both as monotherapy (37) and in combination therapy, e.g., with metformin (38) or pioglitazone (39) and also improves insulin sensitivity (39). DPP-4 inhibitors mimic many of the actions ascribed to GLP-1 in rodents including preservation of β-cell mass through stimulation of cell proliferation and inhibition of apoptosis in rodents (15, 16, 17).

Besides GIP and GLP-1, many gastrointestinal (GI) hormones, neuropeptides, cytokines, and chemokines are substrates for DPP-4 (40), including CXCL10 and SDF1α, both of which are involved in immune regulation, influence the inflammatory response and have been associated with the regulation of β-cell turnover in diabetes (23, 34). Since cytokines and chemokines are crucial for the survival of pancreatic β-cells (41), it remained to be investigated whether DPP-4 inhibitors in culture directly affect β-cell survival and function.

In the present study, the DPP-4 inhibitor linagliptin effectively restored proliferation, survival and β-cell function in a diabetic milieu. The concentrations of 30–50 nM we used were higher than the average c_{max} plasma levels but could be also obtained in patients taking therapeutic doses of linagliptin (42). At all concentrations up to 1 μM, which is 1000-fold the IC_{50} of the human enzyme, linagliptin did not affect β-cell survival at basal conditions in culture.

Human β-cells in culture have only a very limited, if any (42), capacity to proliferate. Here we found 0.4% proliferating β-cells under basal conditions when islets were plated on extracellular matrix coated dishes, and prolif-

eration was further decreased by diabetogenic conditions of chronically elevated glucose, free fatty acids, cytokines or H_2O_2. Linagliptin improved cell survival by decreasing apoptosis, and maintained cells at a steady state level, even under diabetogenic conditions.

The protective linagliptin effect seen in this study was mediated by stabilization of GLP-1 in the islet supernatants, where total as well as active GLP-1 levels were restored and accumulated during islet culture, providing a similar mechanism as seen in vivo.

Active GLP-1 can be produced from islets (43). The α-cells express prohormone convertase PC1/3, which cleaves preproglucagon peptide to generate GLP-1. α-cells are an established islet source of secreted GLP-1 (44, 45, 46, 47) and may provide a local protective paracrine effect for β-cell regeneration under diabetic conditions, especially during β-cell injury (34). Sorted human β-cells also express PC1/3 but the GLP-1 content could not be detected and thus it was assumed that β-cells do not produce GLP-1 (48), although it was found to be secreted from the rodent β-cell line, INS-1E (49).

We found high levels of active GLP-1 (~3 ng/ml from 50 islets over 4 d of culture) were secreted from human islets and accumulated only in the presence of a DPP-4 inhibitor, while high DPP-4 activity could only be measured in the absence of linagliptin.

GLP-1 is accumulated in the medium during the 4-d culture period and provides in turn a paracrine protective effect on the β-cell. Diabetogenic conditions, e.g., the cytokine cocktail IL-1β/IFNγ enhanced IL-6 production. This was counteracted by linagliptin, and paralleled with increased GLP-1 in the medium. It can thus be hypothesized, that higher GLP-1 concentrations in the culture medium in turn inhibits IL-6 production; this would provide a negative feedback loop to the IL-6 effect to induce GLP-1 in islets (44).

Hyperglycemia and a diabetic milieu decrease expression of β-cell GLP-1 receptors in vivo and in vitro (30, 31, 50). In contrast, diabetogenic conditions significantly increased total GLP-1 release from cultured islets. This was also observed in islets from patients with T2D (51) and from diabetic *Psammomys obesus* (47), which show higher GLP-1 secretion than nondiabetic controls.

Slightly reduced GLP-1 secretion after a meal in patients with impaired oral glucose tolerance and more severely impaired GLP-1 secretion in type 2 diabetic patients was observed (52), although controversial results have been obtained in various studies (53). Differences occur between tissue-specific GLP-1 levels and whether total or active GLP-1 was measured (54). It is possible, that reduced GLP-1 in T2D patients is a result of elevated plasma DPP-4 activity (55), which can be induced by chronic hyperglycemia and is paralleled with reductions of active GLP-1 (56).

Linagliptin strongly reduced DPP-4 activity in the human islet supernatant and stimulated GLP-1 stability. Active DPP-4 rapidly degrades SDF-1α (1–68) to inactive SDF-1α (3–68) (57) and thus, more active SDF1α is available upon DPP-4 inhibition. In turn SDF-1α acts like a cytokine and induces its own production (34). This explains the increased SDF-1α mRNA levels with linagliptin treatment, found in the present study.

The regulation of DPP-4 in diabetes has not been fully clarified, both increased (55, 56) and decreased (58) DPP-4 activity have been reported in patients with diabetes. DPP-4 release from adipose tissue is higher in obese individuals and correlates with parameters of the metabolic syndrome, where DPP-4 directly impairs insulin signaling in fat and muscle cells (59). Elevated glucose exposure leads to enhanced DPP-4 activity and mRNA expression in endothelial cells (60). Also in human islets, we detected elevated DPP-4 mRNA in response to elevated glucose and palmitate, while linagliptin inhibited this induction (data not shown). One could speculate that in islets under diabetogenic conditions, there is more DPP-4 produced, which inactivates the elevated GLP-1, and thus it cannot fulfill its functions.

Recent human studies investigating the long-term treatment with vildagliptin on β-cell function were somehow disappointing, because following wash out periods the observed β-cell sparing effects disappeared, thus questioning the disease modifying potential of this drug (6). However, it remains to be elucidated whether this is caused by a limited tissue penetration in vivo or limited availability of the drugs in islets.

In summary, we show that linagliptin has a protective effect on β-cell turnover and function *under diabetogenic conditions*, which is mediated through stabilized GLP-1 and an anti-inflammatory/antioxidative stress pathway.

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Disclosure summary: T. Klein and D.M. Schumann are employees of Boehringer Ingelheim Pharma. Linagliptin is a Boehringer Ingelheim Pharma product. There are no patents, products in development or other marketed products to declare. All other authors have nothing to disclose.

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References

1. **Mentlein R, Gallwitz B, Schmidt WE.** Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem*. 1993;214:829–835.
2. **Drucker DJ, Nauck MA.** The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*. 2006;368:1696–1705.
3. **Nauck M, Stockmann F, Ebert R, Creutzfeldt W.** Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia*. 1986;29:46–52.
4. **Lambeir AM, Scharpe S, De Meester I.** DPP4 inhibitors for diabetes–what next? *Biochem Pharmacol*. 2008;76:1637–1643.
5. **Elashoff M, Matveyenko AV, Gier B, Elashoff R, Butler PC.** Pancreatitis, pancreatic, and thyroid cancer with glucagon-like peptide-1-based therapies. *Gastroenterology*. 2011;141:150–156.
6. **Foley JE, Bunck MC, Moller-Goede DL, Poelma M, Nijpels G, et al.** Beta cell function following 1 year vildagliptin or placebo treatment and after 12 week washout in drug-naive patients with type 2 diabetes and mild hyperglycaemia: a randomised controlled trial. *Diabetologia*. 2011;54:1985–1991.
7. **Brubaker PL, Drucker DJ.** Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology*. 2004;145:2653–2659.
8. **Li L, El-Kholy W, Rhodes CJ, Brubaker PL.** Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. *Diabetologia*. 2005;48:1339–1349.
9. **Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, et al.** Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes*. 2000;49:741–748.
10. **Bulotta A, Hui H, Anastasi E, Bertolotto C, Boros LG, et al.** Cultured pancreatic ductal cells undergo cell cycle re-distribution and beta-cell-like differentiation in response to glucagon-like peptide-1. *J Mol Endocrinol*. 2002;29:347–360.
11. **Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, et al.** Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia*. 2004;47:806–815.
12. **Cechin SR, Perez-Alvarez I, Fenjves E, Molano RD, Pileggi A, et al.** Anti-Inflammatory Properties of Exenatide in Human Pancreatic Islets. Cell Transplant. 2011
13. **Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, et al.** Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology*. 2003;144:5149–5158.
14. **Lupi R, Mancarella R, Del Guerra S, Bugliani M, Del Prato S, et al.** Effects of exendin-4 on islets from type 2 diabetes patients. *Diabetes Obes Metab*. 2008;10:515–519.
15. **Pospisilik JA, Martin J, Doty T, Ehses JA, Pamir N, et al.** Dipeptidyl peptidase IV inhibitor treatment stimulates beta-cell survival and islet neogenesis in streptozotocin-induced diabetic rats. *Diabetes*. 2003;52:741–750.
16. **Cho JM, Jang HW, Cheon H, Jeong YT, Kim DH, et al.** A novel dipeptidyl peptidase IV inhibitor DA-1229 ameliorates streptozotocin-induced diabetes by increasing beta-cell replication and neogenesis. *Diabetes Res Clin Pract*. 2011;91:72–79.
17. **Pospisilik JA, Stafford SG, Demuth HU, McIntosh CH, Pederson RA.** Long-term treatment with dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulin sensitivity in the VDF Zucker rat: a euglycemic-hyperinsulinemic clamp study. *Diabetes*. 2002;51:2677–2683.
18. **Eckhardt M, Langkopf E, Mark M, Tadayyon M, Thomas L, et al.** 8-(3-(R)-aminopiperidin-1-yl)-7-but-2-ynyl-3-methyl-1-(4-methyl-quinazolin -2-ylmethyl)-3,7-dihydropurine-2,6-dione (BI 1356), a highly potent, selective, long-acting, and orally bioavailable DPP-4 inhibitor for the treatment of type 2 diabetes. *J Med Chem*. 2007;50:6450–6453.
19. **Forst T, Uhlig-Laske B, Ring A, Graefe-Mody U, Friedrich C, et al.** Linagliptin (BI 1356), a potent and selective DPP-4 inhibitor, is safe and efficacious in combination with metformin in patients with inadequately controlled Type 2 diabetes. *Diabet Med*. 2010;27:1409–1419.
20. **Sauter NS, Schulthess FT, Galasso R, Castellani LW, Maedler K.** The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. *Endocrinology*. 2008;149:2208–2218.
21. **Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, et al.** Glucose-induced beta-cell production of interleukin-1beta contributes to glucotoxicity in human pancreatic islets. *JClinInvest*. 2002;110:851–860.
22. **Oberholzer J, Triponez F, Mage R, Andereggen E, Buhler L, et al.** Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. *Transplantation*. 2000;69:1115–1123.
23. **Schulthess FT, Paroni F, Sauter NS, Shu L, Ribaux P, et al.** CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. *Cell Metab*. 2009;9:125–139.
24. **Kaiser N, Corcos AP, Sarel I, Cerasi E.** Monolayer culture of adult rat pancreatic islets on extracellular matrix: modulation of B-cell function by chronic exposure to high glucose. *Endocrinology*. 1991;129:2067–2076.
25. **Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, et al.** Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes*. 2001;50:69–76.
26. **Le Bacquer O, Shu L, Marchand M, Neve B, Paroni F, et al.** TCF7L2 splice variants have distinct effects on {beta}-cell turnover and function. *Hum Mol Genet*. 2011;20:1906–1915.
27. **Ceriello A, Mercuri F, Quagliaro L, Assaloni R, Motz E, et al.** Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia*. 2001;44:834–838.
28. **Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, et al.** Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab*. 2004;89:5535–5541.
29. **Sokolovsky M, Riordan JF, Vallee BL.** Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. *Biochemistry*. 1966;5:3582–3589.
30. **Xu G, Kaneto H, Laybutt DR, Duvivier-Kali VF, Trivedi N, et al.** Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: possible contribution to impaired incretin effects in diabetes. *Diabetes*. 2007;56:1551–1558.
31. **Shu L, Matveyenko AV, Kerr-Conte J, Cho JH, McIntosh CH, et al.** Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with downregulation of GIP- and GLP-1 receptors and impaired beta-cell function. *Hum Mol Genet*. 2009;18:2388–2399.
32. **Serre V, Dolci W, Schaerer E, Scrocchi L, Drucker D, et al.** Exendin-(9–39) is an inverse agonist of the murine glucagon-like peptide-1 receptor: implications for basal intracellular cyclic adenosine 3′,5′-monophosphate levels and beta-cell glucose competence. *Endocrinology*. 1998;139:4448–4454.
33. **Waget A, Cabou C, Masseboeuf M, Cattan P, Armanet M, et al.** Physiological and pharmacological mechanisms through which the

DPP-4 inhibitor sitagliptin regulates glycemia in mice. *Endocrinology*. 2011;152:3018–3029.
34. **Liu Z, Stanojevic V, Avadhani S, Yano T, Habener JF.** Stromal cell-derived factor-1 (SDF-1)/chemokine (C-X-C motif) receptor 4 (CXCR4) axis activation induces intra-islet glucagon-like peptide-1 (GLP-1) production and enhances beta cell survival. *Diabetologia*. 2011;54:2067–2076.
35. **Proost P, Struyf S, Schols D, Durinx C, Wuyts A, et al.** Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. *FEBS Lett*. 1998;432:73–76.
36. **Liu Z, Habener JF.** Stromal cell-derived factor-1 promotes survival of pancreatic beta cells by the stabilisation of beta-catenin and activation of transcription factor 7-like 2 (TCF7L2). *Diabetologia*. 2009;52:1589–1598.
37. **Del Prato S, Barnett AH, Huisman H, Neubacher D, Woerle HJ, et al.** Effect of linagliptin monotherapy on glycaemic control and markers of beta-cell function in patients with inadequately controlled type 2 diabetes: a randomized controlled trial. *Diabetes Obes Metab*. 2011;13:258–267.
38. **Taskinen MR, Rosenstock J, Tamminen I, Kubiak R, Patel S, et al.** Safety and efficacy of linagliptin as add-on therapy to metformin in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled study. *Diabetes Obes Metab*. 2011;13:65–74.
39. **Gomis R, Espadero RM, Jones R, Woerle HJ, Dugi KA.** Efficacy and safety of initial combination therapy with linagliptin and pioglitazone in patients with inadequately controlled type 2 diabetes: a randomized, double-blind, placebo-controlled study. *Diabetes Obes Metab*. 2011;13:653–661.
40. **De Meester I, Durinx C, Bal G, Proost P, Struyf S, et al.** Natural substrates of dipeptidyl peptidase IV. *Adv Exp Med Biol*. 2000;477:67–87.
41. **Maedler K, Dharmadhikari G, Schumann DM, Storling J.** Interleukin-targeted therapy for metabolic syndrome and type 2 diabetes. *Handb Exp Pharmacol*. 2011;257–278.
42. **Portha B, Tourrel-Cuzin C, Movassat J.** Activation of the GLP-1 receptor signalling pathway: a relevant strategy to repair a deficient beta-cell mass. *Exp Diabetes Res*. 2011;2011:376509.
43. **D'aleo V, Del Guerra S, Filipponi F, Boggi U, Marchetti P, et al.** The direct effects of DPP-4 inhibition on isolated human islets include protection from glucotoxicity. *Diabetologia*. 2009;52:S35–S36.
44. **Ellingsgaard H, Hauselmann I, Schuler B, Habib AM, Baggio LL, et al.** Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. *Nat Med*. 2011;17:1481–1489.
45. **Marchetti P, Lupi R, Bugliani M, Kirkpatrick CL, Sebastiani G, et al.** A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets. *Diabetologia*. 2012;55:3262–3272.
46. **Whalley NM, Pritchard LE, Smith DM, White A.** Processing of proglucagon to GLP-1 in pancreatic alpha-cells: is this a paracrine mechanism enabling GLP-1 to act on beta-cells? *J Endocrinol*. 2011;211:99–106.
47. **Hansen AM, Bodvarsdottir TB, Nordestgaard DN, Heller RS, Gotfredsen CF, et al.** Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in Psammomys obesus–an adaptive response to hyperglycaemia? *Diabetologia*. 2011;54:1379–1387.
48. **Kirkpatrick CL, Marchetti P, Purrello F, Piro S, Bugliani M, et al.** Type 2 diabetes susceptibility gene expression in normal or diabetic sorted human alpha and beta cells: correlations with age or BMI of islet donors. *PLoS One*. 2010;5:e11053.
49. **Chepurny OG, Holz GG.** Over-expression of the glucagon-like peptide-1 receptor on INS-1 cells confers autocrine stimulation of insulin gene promoter activity: a strategy for production of pancreatic beta-cell lines for use in transplantation. *Cell Tissue Res*. 2002;307:191–201.
50. **Cheong YH, Kim MK, Son MH, Kaang BK.** Glucose exposure pattern determines glucagon-like peptide 1 receptor expression and signaling through endoplasmic reticulum stress in rat insulinoma cells. *Biochem Biophys Res Commun*. 2011;414:220–225.
51. **Marchetti P, Lupi R, Bugliani M, Kirkpatrick CL, Sebastiani G, et al.** A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets. *Diabetologia*. 2012;55:3262–3272.
52. **Nauck MA, Vardarli I, Deacon CF, Holst JJ, Meier JJ.** Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: what is up, what is down? *Diabetologia*. 2011;54:10–18.
53. **Meier JJ, Nauck MA.** Is the diminished incretin effect in type 2 diabetes just an epi-phenomenon of impaired beta-cell function? *Diabetes*. 2010;59:1117–1125.
54. **Mussig K, Staiger H, Machicao F, Haring HU, Fritsche A.** Genetic variants affecting incretin sensitivity and incretin secretion. *Diabetologia*. 2010;53:2289–2297.
55. **Ryskjaer J, Deacon CF, Carr RD, Krarup T, Madsbad S, et al.** Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbAlc levels, but is not acutely affected by food intake. *Eur J Endocrinol*. 2006;155:485–493.
56. **Mannucci E, Pala L, Ciani S, Bardini G, Pezzatini A, et al.** Hyperglycaemia increases dipeptidyl peptidase IV activity in diabetes mellitus. *Diabetologia*. 2005;48:1168–1172.
57. **Hocher B, Reichetzeder C, Alter ML.** Renal and Cardiac Effects of DPP-4 Inhibitors - from Preclinical Development to Clinical Research. *Kidney Blood Press Res*. 2012;36:65–84.
58. **Meneilly GS, Demuth HU, McIntosh CH, Pederson RA.** Effect of ageing and diabetes on glucose-dependent insulinotropic polypeptide and dipeptidyl peptidase IV responses to oral glucose. *Diabet Med*. 2000;17:346–350.
59. **Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, et al.** Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. *Diabetes*. 2011;60:1917–1925.
60. **Pala L, Mannucci E, Pezzatini A, Ciani S, Sardi J, et al.** Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells. *Biochem Biophys Res Commun*. 2003;310:28–31.

Siglec-7 is down-regulated in inflamed islets and activated peripheral blood mononuclear cells; restores β-cell function and survival

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Siglec-7 is down-regulated in inflamed islets and activated peripheral blood mononuclear cells; restores β-cell function and survival

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Abstract

Chronic inflammation is consequential to the etiology of both T1D and T2D. Cytokine and chemokine production by infiltrating macrophages and by β-cells themselves in a diabetic milieu contributes to their destruction in inflamed islets and thus to progression of diabetes. In order to find potential targets for inhibition of this deleterious response of islets, the investigation of underlying mechanisms for triggering inflammation is essential.

The expression of a novel family of adhesion molecules called Sialic acid-binding immunoglobulin-like lectins (Siglecs) in pancreatic islets was observed in a cell type specific manner. Siglec-7, expressed on the β-cells, was down-regulated in diabetes. Over-expression of Siglec-7 in cultured isolated islets prevented β-cell dysfunction and apoptosis under chronic diabetic stimuli and also in diabetic islets. The protective effect of Siglec-7 was mediated by the inhibition of the NF-κB pathway and the subsequent decrease in cytokine secretion. Also, activated immune cells showed loss of Siglec-7 expression. Ultimately, restoration of Siglec-7 in stressed islets caused a reduction in the number of recruited migrating monocytes.

Siglec-7 expression on β-cells contributes to the inhibition of pro-inflammatory activation of these cells in diabetes. Restoration of Siglec-7 expression or signaling may be a potential therapeutic strategy to preserve β-cell function and mass in the manifestation of diabetes. This strategy would not only rescue the β-cells, but also inhibit systemic inflammation observed in T2D.

Introduction

Diabetes mellitus is a syndrome of disordered glucose metabolism, usually due to a combination of hereditary and environmental causes, resulting in hyperglycemia. The ability of the β-cells to secrete adequate amounts of insulin to maintain normoglycemia depends on their function and mass. In both, Type 1 diabetes mellitus (T1D) and Type 2 diabetes mellitus (T2D), the major mechanism leading to decreased β-cell mass is increased β-cell apoptosis. T1D results from an absolute insulin deficiency due to the autoimmune destruction of the insulin producing β-cells [1]. β-cell destruction occurs through immune mediated processes such as mononuclear cell infiltration in the pancreatic islets and interaction between antigen presenting cells and T cells, which leads to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other inflammatory products, and subsequently to β-cell apoptosis. T2D is characterized by chronic insulin resistance and a progressive decline in β-cell function and mass. Obesity is strongly associated with the development of insulin resistance [2], and is the main risk factor for the development of T2D. A chronic, low-grade inflammatory state is present in obesity, with adipose tissue macrophage infiltration and pro-inflammatory activity of macrophages [3]. Epidemiological studies suggest that low-grade inflammation precedes and predicts the development of T2D [4]. Cytokines and chemokines are produced and secreted not only by activated infiltrating macrophages, but also by adipocytes and pancreatic β-cells themselves. Chronic elevated glucose and free fatty acid levels occurring in diabetes trigger pro-inflammatory responses in several tissues like adipose tissue, muscle, liver, immune cells and also the islets [5]. Pro-inflammatory cytokines can cause insulin resistance [6], impair β-cell function [7], and anti-inflammatory mediators may reverse both [8, 9], implying that inflammation may be directly involved in the pathogenesis of T2D. Hence, activation of the innate immune system and triggering of local as well as systemic inflammation are hallmarks of both T1D and T2D.

In the event of immune system activation, the signaling and activation of immune cells is brought by secreted stimulators as well as via cell-cell interactions. Different cell surface receptors and adhesion molecules play a role in the immune system activation. One such family of adhesion and signaling molecules are Sialic

acid-binding immunoglobulin-like lectins (Siglecs) [10]. Siglecs are I-type lectins which recognize and interact via immunoglobulin (Ig)-like domains with sialylated glycan residues on same cell surface (*cis*-interaction) or on neighboring cell surface, extracellular matrix protein or secreted glycoproteins (*trans*-interactions). The siglecs can be divided into two groups: an evolutionarily conserved subgroup (Siglecs-1, -2, -4 and -15) and a rapidly evolving CD33/Siglec- 3-related subgroup (Siglecs-3, -5 to -11 and -14, -16 in primates) [11]. Only Siglec-4 (myelin-associated glycoprotein, MAG) expressed on glial cells and placental Siglec-6 are siglecs present on non-hematopoetic cells. All other siglecs are expressed on the hematopoietic and immune cells, in a very cell-type specific manner. Every siglec recognizes specific sialic acid linkages, thus hinting towards their unique function [12]. Typically, cytoplasmic motifs of siglecs show presence of one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These ITIMs recruit tyrosine phosphatases and eventually can inhibit activatory signals of other receptors. These cell-cell signaling interactions play a role in the immune system [13].

To verify the presence and function of siglecs in the infiltrating immune cells in islets, we investigated expression levels of siglecs in the pancreas. Surprisingly, these investigations have brought to surface the presence of siglecs in non-inflammatory cells in pancreatic tissue, opening a whole new perspective for the role of siglecs in general. Here, we show cell type specific siglec expression in the human endocrine pancreas. Because of its specific presence on β-cells, we focused our experimentation on Siglec-7. Cloned for the first time in 1999 [14], Siglec-7 is a CD-33 related siglec constitutively expressed on all natural killer (NK) cells, monocytes and also on a subset of T cells [15]. Structurally, it is characterized by 3 immunoglobulin-like extracellular domains (one NH 2-terminal V-type and two C2-type), a trans-membrane region and a cytoplasmic tail containing two tyrosine residues located in immunoreceptor tyrosine-based inhibitory motifs. Siglec-7 acts as an inhibitory receptor in human NK cells after engagement by antibodies [14] or binding with sialic acid-containing ligands [16]. Upon phosphorylation, it can recruit the SH2 domain-bearing protein tyrosine phosphatase (PTP) SHP-1 [14]. Anti-Siglec-7 Abs also inhibits the proliferation of myeloid cells [17]. Also, Siglec-7 inhibits the FcRI-mediated serotonin release from RBL cells following crosslinking. The ITIMs are essential for this inhibitory function,

and facilitate tyrosine phosphorylation and recruitment of SHP-1 and SHP-2 phosphatases [18]. Siglec-7 is also expressed on a subset of T-cells and negatively regulates T-cell receptor (TCR) signaling [19]. Thus, Siglec-7 can be considered as an inhibitory receptor, participating in the regulation of cell function and survival.

The present study identifies the role of cell adhesion molecules, siglecs, in the manifestation and progression of T2D. We investigated, whether inhibitory signals by Siglec-7 can restore β-cell survival and function in a diabetic milieu and whether Siglec-7 expression can influence immune cell migration.

Methods

Islet culture. Human islets were isolated from ten pancreata of healthy and 3 diabetic organ donors at the University of Lille or University of Leiden and cultured in CMRL-1066 medium (Invitrogen) as described previously [20]. Briefly, islets were cultured on extracellular matrix coated dishes derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel) for 2 days, allowing the cells to attach to the dishes and spread [21]. They were exposed to 5.5, 22.2 or 33.3 mM glucose, with or without 0.5 mM palmitate (dissolved as described previously [22]) or the mixture of 2 ng/ml recombinant human IL-1β (R&D Systems, Minneapolis, MN) +1,000 U/ml recombinant human IFN-γ (PeProTech, Rocky Hill, NJ, USA) for 72h.

Transfection. At 2 days post-isolation and culture on extracellular matrix coated dishes, isolated islets were transfected using Ca^{2+}-KRH medium (KCl 4.74 mM, KH_2PO_4 1.19 mM, $MgCl_26H_2O$ 1.19 mM, NaCl 119 mM, $CaCl_2$ 2.54 mM, $NaHCO_3$ 25 mM, HEPES 10 mM). After 1h incubation lipoplexes (Lipofectamine2000, Invitrogen, Carlsbad, CA, USA)/DNA ratio 2.5:1, 5 μg CMV-Siglec-7 (Life technologies) or LacZ/GFP control plasmid DNA/100 islets or 100 nM siRNA to Siglec-7 (ON-TARGETplus SMARTpool against human *Siglec-7*, (Dharmacon, Lafayette CO, USA) and scramble siRNA (Dharmacon) were added to transfect the cells as described previously [23, 24]. After additional 6h incubation, CMRL 1066 medium containing 20% FCS and L-Glutamine were added to the transfected islets. Transfection efficiency was determined using RT PCR.

Glucose stimulated insulin secretion. Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated

plates. For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Kreb's Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose (stimulated). Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Islet insulin was determined using mouse insulin ELISA (ALPCO, Salem, NH, USA).

RNA extraction and RT-PCR analysis. Total RNA was isolated from cultured human islets as described previously [20]. For gene expression analysis of siglecs, semi-quantitative Real Time-PCR was performed in the StepOne Plus Real Time PCR system (Applied Biosystems, Darmstadt, Germany) using Power SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt,Germany). cDNA based on RNA from human pancreatic tissues were analyzed for the genes cyclophilin, glucagon, SAT2, insulin and SN1. Amplification of the endogenous housekeeping gene cyclophilin as well as the genes glucagon, SAT2, insulin and SN1 consisted of an initial denaturation step at 95°C for 10 min, followed by 40 PCR cycles of denaturation by 95°C for 30 s, primer annealing by 60°C for 20 s, and elongation by 72°C for 10 s. All Siglecs were amplified carrying out the touchdown PCR with annealing temperatures from 57°C-53°C in each 5 cycles. Primers used for this RT-PCR were: (Siglec-7) 5'AAGAAGCCACCAACAATGAG3'/5'CAGTTAGACAAGAGGAATAAGTTC3'; (Siglec-3) 5'TGGTGTGACTACGGAGAG3'/5'ATGAAGAAGATGAGGCAGAG3' (Siglec-10) 5'CATTATGCCACGCTCAAC3'/5'TCTTCAACCTCTTACTCTACC3'; (insulin) 5'CTACCTAGTGTGCGGGGAAC3'/5'GCTGGTAGAGGGAGCAGATG3'; (glucagon) 5'CATTCACAGGGCACATTCAC3'/5'CAGCTTGGCCTTCCAAATAA3'; (SN1) 5'TACGACGTGCTATCCAGCAG3'/5'CCAGGATTTTAGGGGTGGAT3'; (SAT2) 5'AGTTGCCTTTGGTGATCCAG3'/5'CAGGACACGGAACCTGAAAT3' and (PPIA) 5'TACGGGTCCTGGCATCTTGT3'/5'CCATTTGTGTTGGGTCCAGC3'.

For analysis of PBMCS and isolated islets, we used the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with a commercial kit (TaqMan(R) PCR Master Mix; Applied Biosystems). TaqMan(R) Primers used: Siglec-7 (Hs00255574_m1); Siglec-3 (Hs00233544_m1); ST8SIA1 (Hs00268157_m1); PPIA (Hs99999904_m1); CD25 (Hs00907779_m1); Neu3 (Hs00198406_m1). To analyze the relative changes in the gene expression the

comparative $\Delta\Delta C_t$ method was used. According to this method C_t values of the genes of interest were normalized the reference genes. The resulting ΔC_t values of any sample were adjusted then to a ΔC_t value of the control sample, using the formula $2^{-\Delta\Delta Ct}$ in which $\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$ and $\Delta\Delta Ct = \Delta C_{t,sample} - \Delta C_{t,control}$.

Western Blot analysis. At the end of the incubation periods, islets were washed in ice-cold PBS and lysed in 40 μl lysis buffer RIPA (20 mM Tris acetate, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate) by repeated rounds of freezing and thawing on ice. Prior to use, the lysis buffer was supplemented with Protease- and Phosphatase-inhibitors (Pierce, Rockford, IL, USA). Protein concentration was measured using BCA assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein from each treatment group were run on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and electrically transferred onto PVDF membranes. Membranes were incubated with rabbit anti- p-IκBα; rabbit anti- p-p65 rabbit anti-β-actin (Cell Signaling Technology, Danvers. MA, USA) antibodies, followed by horseradish-peroxidase-linked anti-rabbit IgG. Membrane was developed using a chemiluminescence assay system (Pierce) and analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA, USA). Densitometric analysis of the blots was carried out using Vision Works LS Image Acquisition and Analysis software Version 6.8 (UVP BioImaging Systems, Upland, CA, USA). The gray scale values were normalized on the housekeeping genes as loading controls, and the fold change against control condition was plotted.

Immunocytochemistry. Pancreas from 5 healthy controls and from 5 patients with T2DM were obtained from the National Disease Research Interchange (NDRI), approval for the studies were granted by the Ethical Commission of Bremen University. The tissues were fixed in 4% para-formaldehyde overnight and embedded in paraffin. Islets cultured in suspension were washed with PBS and were fixed in Bouin's (Sigma, Hamburg, Germany) solution for 15 min, resuspended in 2% melted agarose in phosphate buffered saline (PBS), followed by short centrifugation and paraffin embedding. Both, islet agarose pellets and human tissue samples were washed overnight in 70% ethanol followed by dewatering in ethanol and xylol and paraffin embedding using Leica TP1020 tissue

processor (Leica, Microsystems, Wetzlar, Germany). 4µm sections were cut using a microtome and mounted on slides. For immunohistochemical analysis of pancreatic and islet sections, they were deparaffinized and rehydrated by washing twice in toluene for 10 min, respectively, in 100%, 95% and 70% ethanol for 3 min, and then in water for 5 min. Slides were then exposed to antigen-retrieval using Antigen Unmasking Solution (Vector Laboratories, Inc. Burlingame, CA) pre-warmed in a microwave at 600 Watt for 3 cycles each 5 min. and 1 min break in between each cycle. The sections were then cooled to room temperature and permeablized in soaking buffer (0.4% Triton X-100 in TBS) for 30 min. After this, to minimize unspecific binding of antibodies, slides were incubated in blocking buffer containing 0.2% Tween 20, 3% IgG-free Bovine serum albumin (BSA), and 0.5% Triton X-100 or 1 h RT. For Fc-chimera staining, Human Fc receptor blocking reagent (MACS #130-059-901) was used for 15-20 min room temperature. As a negative control for staining with Fc chimera, slides were treated with 10mU/ml *V. cholerae* sialidase (Roche) for 2h at 37°C. Primary antibodies were diluted (1:50 or 1:100) in the antibody dilution buffer (0,2% Tween 20, 3% IgG-free BSA in TBS) as recommended and incubated with the slides for either 1 h room temperature or overnight at 4°C. Antibodies used were: polyclonal rabbit anti-Siglec-7 (Abcam, Cambridge, UK); rabbit anti-human CD22 (Abcam, Cambridge, UK); rabbit anti-human Sialoadhesin (Abcam, Cambridge, UK); monoclonal mouse anti- Siglec-7 (kindly provided by Prof. Paul Crocker), polyclonal sheep anti-Siglec-3,-5,-8,-7 and -10 (kindly provided by Prof. Paul Crocker); Siglec-7 Fc-chimera (kindly provided by Prof. Sørge Kelm); GD3 (R24; Abcam); guinea pig anti-insulin and mouse anti-glucagon. Secondary antibodies were against the primary antibody species and were either FITC- , Cy3- or AMCA-conjugated antibodies (Dako, Hamburg, Germany). For bright field staining, the secondary antibodies conjugated to enzyme alkaline phosphatase were used, followed by development with BCPI/NBT substrate (Sigma, Steinheim, Germany) for 15 min at room temperature. After the staining procedures, slides were mounted with Vectashield with 4'6-diamidino-2-phenylindole (DAPI) (Vector Labs) or Glycerogelatin. Fluorescence was analyzed using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon). Intensity and saturation of the staining was measured using Adobe Photoshop© Extended analysis software after an adapted model used

by Pham et al [25]. Briefly, the insulin positive area in the green channel was manually marked and the selection was saved. By loading this selection onto the red channel image, the area positive for Siglec-7 or GD3 was assigned and measurements were recorded. The mean gray scale values were termed as the saturation and the integrated density values considered as intensities.

For detection of β-cell apoptosis and proliferation, insulin and TUNEL (In Situ Cell Death Detection Kit -AP; Roche Diagnostics, Mannheim, Germany) or Ki67 staining (Mouse anti-Ki67 (7B11) prediluted, Invitrogen, Camarillo, CA, USA) were performed as described previously [26]. Dishes were analyzed using Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon).

Cytokine quantification. The cell culture supernatants stored at -20°C were evaluated in a cytokine multiplex array system called Meso Scale Discovery® (Gaithersburg, MD, USA) using a kit (Human Pro-inflammatory II 4-plex assay) read at the Sector Imager 6000® as per manufacturer's instructions. The platform uses electrochemiluminescence technology, in which multiple specific capture antibodies are coated at corresponding spots on an electric wired microplate. The detection antibody is conjugated to a tag which is excited with emission beams in the electric field applied by the reading instrument. Co-reactants present in the "read buffer" amplify these electric signals [27]. Using a standard curve these signals are quantified and expressed as absolute concentrations.

PBMC isolation. The isolation of PBMCs from buffy coats was adapted from Repnik U et.al.[28]. Briefly**,** buffy coats were obtained from the Central Institute for Transfusion Medicine, Hamburg (Germany) and WBCs were Purified using a Ficoll gradient (GE healthcare, Uppsala, Sweden), a subsequent hyperosmotic Percoll gradient (GE healthcare, Uppsala, Sweden) led to separation of monocytes from lymphocytes and a third iso-osmotic Percoll gradient to monocytes from platelets and dead cells. The pellet obtained after this gradient is the monocyte-enriched fraction, which we refer to as PBMCs. According to the forward and side scatter plots, this fraction contains about 55-80% monocytes along with 45-20% of lymphocytes.

Flow cytometry. The PBMC fraction was cultured in RPMI supplemented with 10% FCS, 2 mM L-Glutamine and 100 U/ml Penicillin-Streptomycin for 12 hours with or without 22.2 mM glucose and 0.5 mM Palmitate. For flow cytometry of

these PBMCS, they were fixed in freshly-prepared in 1% para-formaldehyde for 10 min at RT. After washing, cells were incubated in polyclonal rabbit anti-Siglec-7 Ab (Abcam) followed by incubation with FITC/Alexa 488 labeled donkey anti-rabbit secondary antibody (Dako, Hamburg, Germany). Rabbit IgG was used as isotype control. For CD25 and CD14 labeling, the PE conjugated anti-CD25 Antibody (Beckman and Coulter A0 7774) and PE-Cy5 conjugated anti-CD14 (Beckman and Coulter A0 7765) were incubated for 30 min at 37°C. The fractions analyzed by FACS were: unstained, control with only secondary, single labeled Siglec-7, CD14 and CD25 and a triple stained fraction. Statistical analysis has been performed on the cell populations in all the different quadrants of the dot plots of CD14 vs. Siglec-7 and CD25 vs. Siglec-7 in the triple stained samples, and the data represented in the graphs signify quantifications of the co-stained populations.

Migration assay. After processing buffy coats through a single Ficoll gradient, the purified leukocyte fraction is plated on cell culture inserts with 0.1 µm pore size membranes, placed in 24 well plates (BD biosciences). The lower chamber contained 1:10 diluted conditioned media obtained from transfected and treated islets and migration was allowed at 37°C for 4 hours. Post the duration of migration, the membrane was cut off and mounted on slides in fluorescein di-acetate solution which renders live cells fluorescent. The slides are analyzed with a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired and quantified using NIS-Elements software (Nikon).

Statistical analysis. Samples were evaluated in a randomized manner by G.D, who was blinded to the treatment conditions. Data are presented as means +/- SE and were analyzed by Student's *t*- tests.

Results

Siglecs are differentially expressed in human pancreatic islets

Siglecs are classically expressed in the cells of the hematopoietic system and regulate the inflammatory cell response. Since many pattern recognition receptors and cytokine receptors are highly expressed on β-cells, we assessed expression levels of the 10 classical siglecs in the human pancreas. Immunofluorescent labeling of siglecs, insulin and glucagon revealed the presence of siglecs

predominantly in the endocrine pancreas. The evolutionarily conserved siglecs Siglec-1 (Sialoadhesin) and Siglec-2 (CD22); as well as Siglec-7 and -10 were expressed exclusively in the β-cells (Fig.1A-D) and Siglec-3,-5, and -8 were expressed solely by the α-cells (Fig.1E-G). In contrast, Siglec-4, -6 and -9 were not expressed in the pancreatic islets. The cellular localization was confirmed by carrying out Confocal Laser Scanning Microscopy (data not shown). Cell surface expression of Siglec-7 in islets was confirmed by flow cytometric analysis of dispersed islets (Supp. Fig. 1A). In addition to this, the presence of Siglec-7 was tested using two different polyclonal antibodies and a monoclonal antibody (data not shown), which validated our observations in human pancreas sections.

Siglec-7 and -3 are oppositely regulated in type 2 diabetes

In order to understand the role of siglec expression in the endocrine pancreas, we investigated whether siglecs are regulated in T2D. Semi-quantitative real time PCR analysis was performed on cDNAs obtained from non-diabetic and diabetic human pancreas from autopsy. In addition to housekeeping genes, expression levels of siglecs were normalized on cell specific markers of β- and α-cells i.e. insulin and glucagon, to account for the changes in their mass in diabetic individuals. In addition, pancreatic siglec expression was normalized to the β- and α-cell specific glutamate receptors SN1 and SAT2, whose expression is unregulated in diabetes [29]. Siglec-7 expressed on β-cells was drastically decreased in diabetic individuals when normalized on expression levels of cyclophylin (PPIA), insulin and SN1 (Fig.2A; reduced by 94%, 85%, 94% respectively in individuals with T2D). Also, Siglec-10 was significantly down-regulated in T2D as compared to cyclophylin (PPIA) and SN1 and showed similar tendency when normalized on insulin (Supp.Fig.1B). On the other hand, the α-cell specific Siglec-3 showed substantial increase in diabetes upon normalization with cyclophylin (PPIA), glucagon and SAT2 (Fig.2A; induced to 5.15-, 4.29-, 5.52-fold, respectively in individuals with T2D, vs. non-diabetic controls). A decrease in insulin mRNA was confirmed in T2D (Fig.2B), while glucagon mRNA showed an increase in T2D (Fig.2C) and β- and α-cell specific SN1 and SAT2 remained unchanged in T2D (Fig.2D, E).

The down-regulation of β-cell siglecs was confirmed in freshly isolated human islets from organ donors with T2D and controls. Siglec-7 showed 87% reduction vs non-diabetic control islets (Fig.2F) and Siglec-10 showed a similar decrease

(Suppl.Fig.1C). Because of the β-cell specific expression and significant regulation in diabetes, we focused our research on the presence and implication of Siglec-7 in the progression of diabetes.

Siglecs bind to different linkages of the terminal sialic acid to its underlying glycan with varying affinities [30]. Siglec-7 has a binding preference for α2,8-linked disialic acid, which leads to downstream signaling via its cytoplasmic inhibitory motifs [31]. In contrast to Siglec-7, the sialyl-transferase responsible for α2-8 linkage formation, St8Sia1 showed a tendency of up-regulation in the diabetic islets (Fig.2G), suggestive of a compensatory mechanism. The membrane-associated sialic acid-cleaving enzyme sialidase Neu3 (Fig.2H), which may unmask Siglec-7 residues and thus induce Siglec-7 mediated inhibition of cell death [16], was significantly down-regulated in T2D islets, which is a further deleterious signal in the inflammation-initiation cascade.

GD3 is one of the endogenous ligands for Siglec-7 which displays 2,8-linked disialic acids [32]. High levels of GD3 reverse the Siglec-7 protective functions in cell survival [16]. Its constitutive expression was detected in β-cells in human pancreas sections by immunofluorescent labeling. As compared to non-diabetic individuals GD3 was more strongly expressed in patients with T2D. Quantification of the staining showed a 1.50-fold and 3.27-fold increase in intensity and saturation respectively in non-diabetic controls vs. patients with T2D (Fig.2I, J). The loss in Siglec-7 expression was confirmed in the same sections from autopsy and showed a 60% and 63% decrease in intensity and saturation respectively in patients with T2D vs. non-diabetic controls (Fig.2K, L).

We further confirmed the opposite regulation of Siglec-7 ligands by another biochemical approach. Chimeric proteins consisting of the IgG like V-set domain attached to an Fc-region were constructed, expressed and purified from the Cho-lec1 cell line [33]. They were used as probes to detect the presence of their binding partners. As a negative control, slides treated with sialidase were probed, in which there was no detection of ligands. Bright field staining of the chimeras revealed the presence of Siglec-7 ligands in both α- and β-cells (Fig.2M), which was increased in pancreatic sections of patients with T2D. These findings hint towards a disruption of Siglec-7 engagement in diabetic islets, and the cells attempt to counteract this by up-regulating its ligands.

Siglec-7 over-expression improves β-cell survival and function

In order to understand the physiological impact of decreased Siglec-7 expression in diabetes, we used an *in vitro* model of human islets exposed to a diabetic milieu of elevated glucose (22.2-33.3 mM) (HG) and free fatty acid (0.5 mM palmitate)(Pal) levels or the cytokine mixture of IL-1β and IFNγ (IL/IF) and subsequently investigated whether re-expression of Siglec-7 can restore β-cell function and survival under diabetogenic conditions. Siglec-7 was restored by liposome-mediated transfection of human islets isolated from non-diabetic and diabetic (T2D) organ donors. Plasmid over-expression of Siglec-7 was analysed using flow cytometry following over-expression in HEK293T cells (Supp.Fig.1D).

β-cell function (Fig.3A-D) and survival (Fig.3E-F) were impaired by all diabetogenic culture conditions (Fig.3A,C,D) as well as in islets isolated from patients with T2D (3B,D,F). In contrast, Siglec-7 over-expression improved β-cell function and survival at all diabetic conditions (3A-F) in healthy non diabetic islets as well as in islets isolated from T2D organ donors, where Siglec-7 over-expression completely normalized β-cell function and survival in these islets (Fig. 3B, D and F). Treatment of diabetic islets with a diabetic milieu did not further impair the β-cells.

In line with our observation in the diabetic pancreas, diabetogenic conditions *in vitro* led to a loss in Siglec-7 expression (Fig.3G). Down-regulation of Siglec-7 by siRNA shows that a loss of Siglec-7 alone impairs β-cell function *in vitro* (Fig.3H, I; 60% reduction in GSIS, as compared to scramble transfected control islets). Furthermore, the deleterious effects of glucolipotoxicity and cytokines impair insulin secretion in β-cells; loss of Siglec-7 adds to it as seen by a tendency of lower stimulatory indices in cytokine treated islets. This down-regulation may directly influence β-cell survival and function under these conditions. We checked for possible mechanisms by which Siglec-7 can bring about inhibition of destruction of β-cells.

Siglec-7 over-expression inhibits NF-κB activation and cytokine production

Islets exposed to elevated glucose and palmitate or cytokines are triggered to secrete pro-inflammatory cytokines. As Siglec-7 is an inhibitory cell adhesion molecule on immune cells [34], we hypothesized that the protective role of Siglec-7 was mediated via the inhibition of inflammation. Expression and secretion of both IL-1β and IL-6 were induced by exposure of islets to elevated glucose and

palmitate as well as by the cytokine mixture; this was strongly inhibited on islets over-expressing Siglec-7 (Fig. 4A-D).

In order to identify the underlying signaling cascades of Siglec-7 mediated β-cell protection, downstream inflammatory pathways were investigated. Western blot analysis of isolated islets treated with elevated glucose and palmitate and the cytokine mixture revealed the activation of the NF-κB pathway, as observed by the induction of phosphorylation of IκB-α and p65 phosphorylation at Ser536, both core components leading to NF-κB activation (Fig.4E,F). This induction could at least be partially prevented by over expression of Siglec-7 shown by subsequent decrease in IκB-α phosphorylation (Fig.4E, H, I). p65 phosphorylation at Ser536 merely enhances its transcription factor activity and is known to be independent of IκB-α [35]. Nevertheless, also the induction of p65 phosphorylation upon cytokine treatment was inhibited upon Siglec-7 over-expression (Fig.4F, J, K). These observations consolidated the immunosuppressive role of Siglec-7 in the prevention of triggering islet inflammation observed in diabetes.

Since also PBMCs showed induction of phosphorylation of IκB-α and p65 phosphorylation at Ser536 in response to elevated glucose and palmitate and the cytokine mixture IL/IF (Fig.4G), we subsequently analyzed Siglec-7 expression in PBMCs under a diabetogenic milieu.

Loss of Siglec-7 is a hallmark of LPS and Gluc/Pal mediated monocyte activation.

Since Siglec-7 is classically expressed by cells of the immune system, we investigated whether diabetogenic conditions would also affect Siglec-7 expression in enriched monocytes. The monocyte-enriched fraction of peripheral blood mononuclear cells (PBMCs) was isolated from human buffy coats using sequential Ficoll-Percoll gradients and exposed to control media, Lipopolysaccharide (LPS, 20 µg/ml) as known activator of the immune cells or the mixture of 22.2 mM glucose and 0.5 mM palmitate. The activated state of these cells was analyzed at 2h and 12h after treatment by real time PCR analysis of the expression of CD25 and the cytokine IL-6. The activation of the canonical NF-κB pathway in PBMCs after the chronic treatments was seen by western blot analysis of the cell lysates (Fig.4G). LPS elucidated rapid (after 2h) and sustained (after 12h) activation of immune cells, as seen by the induction of IL-6 expression after 2h exposure (Fig.5A, B; ~22-fold/~390-fold at 2 and 12h as compared to control).

Elevated glucose/palmitate induced a similar activation pattern in monocytes but to a lesser extent (Fig.5A, B; ~4-fold/~50-fold at 2 and 12h as compared to control). Both LPS and glucose/palmitate induced the expression of CD25 after 2h and, similar to IL-6, CD25 expression was much higher induced after 12h, with LPS showing a stronger effect (Fig.5C, D; ~45-fold, ~3-fold at LPS and Gluc/Pal respectively, as compared to control). In parallel, Siglec-7 m-RNA expression was down-regulated already after 2h treatment with LPS and glucose/palmitate (Fig.5E, F; 45% reduction as compared to control). While LPS-induced Siglec-7 down-regulation was only transient, glucose/palmitate induced a sustained Siglec-7 down-regulation (Fig.5F; 47% reduction as compared to control), suggesting the chronic nature of activation of these cells under conditions of glucolipotoxicity. In line with our findings that Neu3 is decreased in islets in T2D, there was marked down-regulation of Neu3 gene expression in the activated PBMCs at both acute and chronic treatments (Fig.5G, H). For confirming the changes in mRNA, we carried out flow cytometric analysis of the treated PBMCs (Fig.5I). The mean fluorescence intensity of Siglec-7 staining of cells treated with LPS and glucose/palmitate was decreased by 21.38% and 28.49%, respectively, as compared to control (Fig.5J), along with increased expression of CD25 (Supp.Fig.3).

To assess the Siglec-7 expression in activated cell population, cell surface expression of CD25 and CD14 was checked along with Siglec-7. By plotting the intensity of Siglec-7 vs intensity of either CD14 or CD25, followed by quadrant analysis, we determined the Siglec-7 intensity in these cells. The CD25 and Siglec-7 population increases upon exposure to LPS or Gluc/Pal, and the Siglec-7 expression in these cells decreased. On the other hand $CD14^{+}$ $Sig7^{+}$ cells showed a decline in these conditions, with Siglec-7 staying stably expressed in these cells. Thus, loss of Siglec-7 expression was observed only in activated PBMCs, indicating the decreased immune-suppression in these conditions (supplementary figure 2).

Immune cell migration into inflamed islets is inhibited by Siglec-7

Increased infiltration of macrophages has been observed in islets in T2D [36]. To elaborate on the immune-regulatory role of Siglec-7, we evaluated the migration of monocytes *in vitro* in response to conditioned media obtained from isolated human islets, which had been exposed to diabetogenic conditions. For this, we

established an *in vitro* migration assay wherein leukocytes isolated from human buffy coats were allowed to migrate over a period of 4h through a 0.1µm membrane to the lower compartment containing the conditioned islet media. The membranes were mounted in fluorescein diacetate solution, which rendered the live cells fluorescent upon excitation and enabled quantification by fluorescence microscopy (Fig.5M). Induced migration of immune cells was observed under conditions of elevated glucose/palmitate (Fig.5K, 9.61-fold as compared to control), which demonstrates the triggering of inflammation in islets upon chronic exposure to elevated glucose/palmitate. Conditioned media from islets overexpressing Siglec-7 majorly inhibited cell migration of immune cells (Fig.5K, 74% reduction as compared to glucose/palmitate-treated, LacZ-transfected control islets). We also analysed the migratory response of the cells towards isolated islets obtained from patients with T2D. Islet supernatants from T2D islets induced significantly higher migration of the immune cells (Fig. 5L, ~ 2.2-fold induction as compared to non-diabetic islet supernatants, $p<0.05$) which could also be blocked by restoring Siglec-7 expression in these islets (Fig. 5L 54%, reduction as compared to lacz-transfected control T2D islets, $p<0.05$). Treatment with glucose and palmitate could not further induce the migration in diabetic islets, but Siglec-7 over-expressing islets showed a decreased tendency of immune cell migration (data not shown). These findings elaborate on the anti-inflammatory role and the inhibition of immune cell stimulation by Siglec-7 in β-cells.

Discussion

The present study investigated the role of sialic acid-binding immunoglobulin-like lectins (Siglecs), a novel, and still-expanding family of cell adhesion molecules, in islet inflammation observed in T2D. Siglecs, previously known to be majorly expressed in the hematopoietic cells, are also expressed, in a cell type specific manner, in the endocrine pancreas. Of the evolutionarily evolving CD-33 related siglecs which were investigated, Siglec-7 and -10 were expressed solely in the β-cells, whereas Siglec-3, -5 and -8 were expressed only in the α-cells. Although Siglec-7 shares around 84% sequence homology with Siglec-9 [37], its presence was undetectable in the endocrine pancreas as assessed by real-time PCR and immunohistochemical analyses. The β-cell specific Siglec-7 and -10 were

markedly down-regulated in pancreas of individuals with T2D. In contrast, Siglec-3 was significantly up-regulated in α-cells in T2D.

Owing to the significant, drastic regulation of Siglec-7 in diabetes, we focused our research on its role in the β-cells. A marked decrease in the surface expression of Siglec-7 is the foremost marker of the aberrant NK-cell dysregulation in patients with chronic HIV-1 viremia [38]. Similarly, we saw down-regulation of Siglec-7 in β-cells, in T2D. Thus, we hypothesized that the loss of Siglec-7 in the β-cells contributed to their dysfunction and apoptosis. Indeed, restoring surface Siglec-7 expression protected the β-cells from deleterious effects of a diabetic milieu. Siglec-7 could not only maintain glucose stimulated insulin secretion even under diabetogenic conditions, but also inhibit β-cell apoptosis. This rescue of function and survival was also evident in *in vitro* studies of freshly isolated T2D islets. Additionally, depletion of Siglec-7 in healthy isolated control islets impaired β-cell function. Hence, we could conclude that loss of Siglec-7 in the β-cells contributed to their destruction observed in T2D. Siglec-3 also has cytoplasmic ITIMs, but its functional significance in α-cells needs to be further investigated. Also, it is one C2-set domain shorter than Siglec-7, and might vary its interactions within the islets.

Siglec-7 has an unusual binding preference for α-2,8-linked disialic acids and weaker interactions with branched α-2,6 sialyl residues [31]. Subsequently, we immunohistochemically investigated the presence of these binding partners in the human pancreas using Siglec-7 Fc-chimeras, and found them to be expressed in islets. Interaction partners were present in both α- and β-cells, indicating possibility of intra-islet *trans* interactions of Siglec-7 with its ligands on both these cell types.

An endogenous ligand of Siglec-7, ganglioside GD3, was strongly up-regulated in diabetes. The disialoganglioside GD3 is an acidic glycosphingolipid, generated downstream of the ceramide-driven ganglioside biosynthesis, by sialylation of its immediate precursor GM3 by the GD3 synthase (α-2,8-sialyltransferase or ST8Sia I or SAT II) [39]. In freshly isolated islets obtained from autopsy of patients with T2D, we could detect a tendency of increase in ST8SiaI expression, which supported our observed up-regulation of GD3 in diabetic individuals. GD3 activates Fas and ceramide mediated apoptosis, directly targets mitochondria and disrupts mitochondrial trans-membrane potential [40], leading to the release of apoptotic factors such as cytochrome c, production of ROS and activation of AIF

and caspase-9 [41]. Its induced expression in diabetes, thus, hints not only towards feedback up-regulation of ligand upon loss of Siglec-7 expression, but also gives an indirect proof of activation of the pro-apoptotic signaling like that of the Fas receptor, previously reported in the context of glucotoxicity as well as immune mediated β-cell destruction in islets [42, 43]. Increased levels of GD3 in serum have also been implicated in inflammatory processes such as atherosclerosis [44] and lipopolysaccharide triggered inflammation in brain, wherein the microglial cells are activated and secrete GD3 leading to apoptosis of oligodendrocytes [45]. Parallels can be drawn between cytokines (e.g. IL-1β) and GD3, as both at low concentrations, stimulate cell proliferation while at high concentration triggers apoptosis [7, 46]. Also, GD3 expression has been observed specifically in the islets of a T1D mouse model i.e. NOD mice, whereas its precursor GM3 is expressed in the wild type islets [47]. Hence, increased GD3 expression reiterates the pro-apoptotic inflamed state of islets in T2D.

While we observed increased GD3 synthase, the membrane-associated sialidase, specific for ganglioside, NEU3 [48] was decreased in islets isolated from individuals with T2D. NEU3 cleaves off the surface sialic acid residues, which can unmask Siglec-7 and induce its inhibitory signaling cascade [16]. This unmasking may be diminished in T2D, as observed by significantly lower levels of NEU3 consequently leasing to increased Siglec-7 ligand expression in diabetic islets.

Tissue specific effects of NEU3 were observed previously; mice over-expressing NEU3 mainly in muscles develop severe insulin-resistant diabetes [49], but, hepatic NEU3 over-expression improves insulin sensitivity and glucose tolerance through modification of ganglioside composition and Peroxisome Proliferator-activated Receptor gamma (PPAR-γ) signaling [50]. In islets, PPAR-γ activation restores β-cell function under conditions of hyperglycemia and cytokine stress [51] and also regulates β-cell transcription factors PDX-1 and Nkx6.1[52]. Decreased NEU3 expression in T2D islets may thus lead to reduced PPAR-γ signaling, and hence might contribute to β-cell dysfunction under diabetogenic conditions.

Activated and insulin resistant immune cells have been observed in obesity [53]. Also, chronic exposure to FFAs leads to activation of monocytes, along with up-regulation of toll-like receptors TLR2 and TLR4 [54], which leads to impaired

glucose metabolism on the level of insulin sensitive as well as insulin producing cells. As Siglec-7 is endogenously expressed mainly by natural killer cells and monocytes and balances the immune response, the observed loss of Siglec-7 in PBMCs was a hallmark of activated monocytes under diabetogenic conditions. Acute exposure to LPS as well as elevated glucose and palmitate was sufficient to inhibit Siglec-7 expression. In spite of that, chronic exposure to LPS led to restoration of the messenger RNA, but maintained reduced cell surface expression of Siglec-7. Glucose and palmitate chronically kept both the mRNA and protein levels down, proving their chronic effect on the activation of the immune cells under circumstances when also IL-6 was induced. CD25, an atypical marker for activated macrophages [55] was induced after chronic treatment with the diabetic milieu, reiterating the activation of these cells.

The down-regulation of Siglec-7 in activated PBMCs goes in hand with the decreased expression in islets under conditions of inflammation, which highlights its anti-inflammatory role in both these cell types. Ultimately, we investigated the effect of restoration of the siglecs in the islets on the actual infiltration of the immune cells.

Maintaining Siglec-7 expression in stressed islets under glucolipotoxic conditions could inhibit the recruitment and migration of the immune cells. The increased number of macrophages per islet observed *in vivo* in diabetes [36] could, for the first time, be confirmed *in vitro* using a leukocyte migration assay, which also be prevented by restoring Siglec-7 expression in these islets. Hence, Siglec-7 expression in islets is essential for maintaining an anti-inflammatory environment in islets, which prevents subsequent immune system activation. Summarizing the findings, we detected the presence of a novel family of cell adhesion molecules-Siglecs, expressed in the endocrine cells of the pancreas. One of its β-cell specific members, Siglec-7 was lost in diabetes. Restoration of Siglec-7 in these cells could protect them from the harmful effects of diabetic milieu, and help preserve β-cell function and survival under these conditions by inhibition of pro-inflammatory cytokine secretion by suppressing NF-κB activity. Not only was this immune-modulatory function evident in the cytokine profile of the β-cells, but also the PBMCs showed loss of Siglec-7 expression upon activation. Taken together, Siglec-7 plays a substantial role in the maintenance of immune-suppressive anti-inflammatory microenvironment, which is lost in diabetes, and may contribute to

the manifestation and progression of this metabolic syndrome (Suppl.Fig.4). Thus, preserving Siglec-7 expression and function on β-cells as well as immune cells may be a novel therapeutic strategy which could help target both, the sensitization and pro-inflammatory activation of the immune system as well as the islets, thereby being beneficial to effectively halt the deterioration of islets in T2D.

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Figure legends

Figure 1. Siglecs are differentially expressed in the human Islets of Langerhans.

Triple immunostaining for insulin (blue), glucagon (green) and siglecs (red) was carried out on human pancreatic sections obtained at autopsy of healthy non-diabetic controls and patients with T2DM, all with documented fasting plasma glucose >150 mg/dl. **(A)** Siglecs 1, **(B)** -2, **(C)** -7and **(D)** -10 were expressed in β-cells. **(E)** Siglecs 3, **(F)** -5, **(G)** -8 were expressed solely in α-cells. Representative analyses from 5 pancreases from patients with T2D and 5 controls are shown.

Figure 2. **Siglec-7 and -3 are oppositely regulated in type 2 diabetes**

Semi quantitative real time PCR analysis was performed on cDNAs obtained from non-diabetic (n=9) and diabetic human pancreas (n=5) from autopsy. **(A)** Siglec-7 expression was normalized on cyclophilin (PPIA), insulin (ins) and SN1; whereas Siglec-3 expression was normalized on cyclophylin, glucagon (gluc) and SAT2. **(B-E)** Insulin; Glucagon; SN1 and SAT2 were normalized on cyclophylin. **(F-H)** Real

time PCR analysis of isolated islets obtained from autopsy of T2D patients (n=5) were compared to that of nondiabetic individuals (n=3) of **(F)** Siglec-7; **(G)** St8Sia1 and **(H)** Neu3 **(I,J)** Immunohistochemical analysis was carried out on human pancreatic sections obtained at autopsy of healthy non-diabetic controls (n=3) and patients with T2DM (n=3) for **(I)** GD3 and **(K)** Siglec-7; **(J,L)** staining saturation and intensity were quantified using Photoshop; each data point represents saturation and intensity of the protein signal of islets from 3 donors, 2 sections per donor, average 7 islets per section. **(M)** Bright field staining using Siglec-7 Fc-chimeras; along with glucagon (red) and insulin (green) of pancreas sections obtained from autopsies of healthy non-diabetic controls (n=3) and patients with T2DM (n=3); along with control slides treated with sialidase treatment. (*$p<0.05$ to 5.5 mM glucose control).

Figure 3. Siglec-7 over-expression improves β-cell survival and function

Freshly isolated human islets of nondiabetic individuals as well as from patients with T2D were cultured on extracellular matrix-coated dishes and exposed to elevated glucose (22.2 or 33.3 mM) (HG) with or without palmitate (HGPal), palmitate alone (Pal) or the cytokine mixture IL-1β (2 ng/ml) and IFNγ (1,000 U/ml) (IL/IF) for 72 h with or without over-expression by lipofectamine-mediated Siglec-7 plasmid transfection. Glucose stimulated insulin secretion assays were performed after the 72h culture period. **(A,B)** Basal (2.8 mM) and glucose stimulated (16.7mM) insulin secretion was expressed as percent change of control condition basal insulin levels. **(C,D)** Stimulatory index denotes the amount of glucose stimulated (16.7 mM glucose) divided by the amount of basal insulin secretion. Fold changes in stimulatory indices of treated islets were plotted, compared to stimulatory index of control islets **(E,F)** Apoptosis was analyzed by the TUNEL assay in dishes. Islets were triple-stained for insulin and counterstained for DAPI (not shown). Results are means ± SE of the percentage of TUNEL-positive β-cells. The average number of β-cells counted was 8124 for each treatment group in 3 separate experiments from 3 separate dishes per treatment from 3 different organ donors. **(G)** Isolated human islets were treated with 22.2 mM glucose and 0.5 mM palmitate; or the cytokine mixture IL/IF, followed by immunohistochemical analysis of paraffin-embedded islet sections. Representative images show glucagon (green), Siglec-7 (red) and DAPI (blue). **(A-D)** Data are shown from parallel experiments as mean ± SE from 3 islet isolations from 3 different donors. For

control islets; *p<0.05 to 5.5 mM glucose treated LacZ transfected islets, **p<0.05 to diabetic stimuli treated LacZ transfected islets For T2D analysis, #p<0.05 to 5.5 mM glucose treated LacZ transfected nondiabetic control islets.

Figure 4. Siglec-7 inhibits NF-κB activation and cytokine secretion.

Human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to elevated glucose (22.2 mM) and palmitate (Gluc/Pal) or the cytokine mixture IL-1β (2 ng/ml) and IFNγ (1,000 U/ml) (IL/IF) for 72 h with or without over-expression by lipofectamine-mediated Siglec-7 plasmid transfection. **(A-D)** The cytokine profiles of the supernatants of transfected and treated islets were assessed using protein microarray ELISAs. Western blot analysis was performed after Siglec-7 over-expression in islets and 72h treatment with **(E)** 22.2mM glucose and palmitate or **(F)** IL1β and IFNγ; and analyzed for p-p65, p-IKBα and actin. **(G)** PBMCs purified from buffy coats were exposed to 22.2mM glucose and palmitate or IL1β and IFNγ for 12h followed by western blotting. The blots were probed for p-p65, p-IκBα and actin. **(H-K)** Densitometry was carried on blots, protein of interest i.e. p- IκBα and p-p65, normalized on housekeeping, and plotted as fold change of islets at control condition. The graphs and blots are representative of 3-4 independent experiments. *p<0.05 to 5.5 mM glucose treated LacZ transfected control islets, **p<0.05 to diabetic stimuli treated LacZ transfected control islets.

Figure 5. Immune cell migration into inflamed islets is inhibited by Siglec-7.

PBMCs purified from buffy coats of blood donors (n=6) were treated with Lipo polysaccharide (LPS) or elevated glucose and palmitate (Gluc/Pal) for **(A,C,E,G)** 2h or **(B,D,F,H)** 12h. Real time PCR analysis of these treated cells was carried out for **(A-B)** IL6, **(C-D)** CD25, **(E,F)** Siglec-7 and **(G,H)** Neu-3. Cell surface expression of Siglec-7 in the treated PBMCs was determined using flow cytometry. **(I)** Histograms for intensity of Siglec-7 (FL1 filter) was plotted and overlayed to observe the effect of these treatments. **(J)** Histograms were quantified and Siglec-7 expression was plotted as % mean fluorescent intensity as compared to untreated control fraction. The migration of leukocytes (n=3 buffy coat donors) in response to conditioned media obtained from transfected and treated islets (n=3 separate dishes from 3 independent experiments from 3 donors), was quantified after 4h using an *in vitro* migration assay. **(K)** The fold induction of migration as compared to untreated control islet supernatants was plotted. **(L)** Migration of

mononuclear cells (n=3) with respect to cultured islets from diabetic donors (n=3), with or without Siglec-7 over-expression, was plotted as fold change of migrated cells compared to untreated control islets of a nondiabetic individual. **(M)**The images are representative of fluorescent microscopic analysis of live cells migrating through membranes observed in green. For PBMC treatments; *p<0.05 to 11.1mM glucose treated monocyte fraction. For migration assay, *p<0.05 to monocyte fraction treated with 5.5 mM glucose, LacZ transfected control islets, **p<0.05 to monocyte fraction treated with Gluc/Pal, LacZ transfected control islets.

Supplementary Figure 1: **(A)** Islets were dispersed using accutase and cultured overnight for recovery. These dispersed cells were stained with Siglec-7 and analyzed by flow cytometry. Histograms indicate the intensity of FL1 for unstained, isotype control and Siglec-7 stained cells. **(B)** Semi quantitative real time PCR analysis was performed on cDNAs obtained from non-diabetic (n=9) and diabetic human pancreas (n=6) from autopsy. Siglec-10 expression was normalized on cyclophilin (PPIA), insulin and SN1 **(C)** Real time PCR analysis for Siglec-10 of isolated islets obtained from autopsy of T2D patients (n=3) were compared to that of nondiabetic individuals (n=3). **(D)** HEK293T cells were transfected with LacZ or Siglec-7 plasmids were stained for Siglec-7 and analyzed using flow cytometry. (*p<0.05 to 5.5 mM glucose control)

Supplementary Figure 2: (A) PBMCs purified from buffy coats of blood donors (n=6) were treated with elevated glucose and palmitate for 12h. They were triple stained for Siglec-7, CD-14 and CD-25 and analyzed using flow cytometry. Their intensities were plotted against each other, and the quadrants were analyzed for number of positive cell and signal intensities. **(B)**The % cells co-labeled for CD25 and Siglec-7 were quantified, and also the **(C)** % mean fluorescent intensities of Siglec-7 in these cells was plotted. **(D)** % cells co-labeled for CD14 and Siglec-7 were quantified, and the **(E)** % mean fluorescent intensities of Siglec-7 in these cells were plotted. *p<0.05 to 11.1mM glucose treated monocyte fraction.

Supplementary Figure 3: Cell surface expression of CD25 in elevated glucose and palmitate treated PBMCs (n=6) after 12h was determined using flow cytometry. Histograms for intensity of CD25 (FL2 filter) were plotted and overlayed

to observe the effect of these treatments. *p<0.05 to 11.1mM glucose treated monocyte fraction.

Supplementary Figure 4: This model illustrates the role of Siglec-7 as investigated in this paper. In healthy individuals, Siglec-7 helps to maintain a pro-survival anti-inflammatory signaling in monocytes as well as β-cells. In diabetes however, chronic elevated glucose along with palmitate and cytokines cause loss of Siglec-7 in these cells. This leads to triggering of apoptotic and pro-inflammatory signals, activation of macrophages and ultimately to β-cell death.

References

1. Tisch, R. and H. McDevitt, *Insulin-dependent diabetes mellitus.* Cell, 1996. **85**(3): p. 291-7.
2. Mokdad, A.H., et al., *Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001.* JAMA, 2003. **289**(1): p. 76-9.
3. Neels, J.G. and J.M. Olefsky, *Inflamed fat: what starts the fire?* J Clin Invest, 2006. **116**(1): p. 33-5.
4. Festa, A., et al., *Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study.* Diabetes, 2002. **51**(4): p. 1131-7.
5. Donath, M.Y., et al., *Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity.* Cell Metab, 2013. **17**(6): p. 860-72.
6. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.
7. Maedler, K., et al., *Glucose-induced beta-cell production of interleukin-1beta contributes to glucotoxicity in human pancreatic islets.* J.Clin.Invest, 2002. **110**: p. 851-860.
8. Larsen, C.M., et al., *Interleukin-1-receptor antagonist in type 2 diabetes mellitus.* N Engl J Med, 2007. **356**(15): p. 1517-26.
9. Yuan, M., et al., *Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta.* Science, 2001. **293**(5535): p. 1673-7.
10. Crocker, P.R., et al., *Siglecs: a family of sialic-acid binding lectins.* Glycobiology, 1998. **8**(2): p. v.
11. Jandus, C., H.U. Simon, and S. von Gunten, *Targeting siglecs--a novel pharmacological strategy for immuno- and glycotherapy.* Biochem Pharmacol, 2011. **82**(4): p. 323-32.
12. Crocker, P.R. and A. Varki, *Siglecs in the immune system.* Immunology, 2001. **103**(2): p. 137-45.
13. Crocker, P.R., J.C. Paulson, and A. Varki, *Siglecs and their roles in the immune system.* Nat Rev Immunol, 2007. **7**(4): p. 255-66.
14. Falco, M., et al., *Identification and molecular cloning of p75/AIRM1, a novel member of the sialoadhesin family that functions as an inhibitory receptor in human natural killer cells.* J Exp Med, 1999. **190**(6): p. 793-802.

15. Nicoll, G., et al., *Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes.* J Biol Chem, 1999. **274**(48): p. 34089-95.
16. Nicoll, G., et al., *Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms.* Eur J Immunol, 2003. **33**(6): p. 1642-8.
17. Vitale, C., et al., *Engagement of p75/AIRM1 or CD33 inhibits the proliferation of normal or leukemic myeloid cells.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15091-6.
18. Avril, T., et al., *The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells.* J Immunol, 2004. **173**(11): p. 6841-9.
19. Ikehara, Y., S.K. Ikehara, and J.C. Paulson, *Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9.* J Biol Chem, 2004. **279**(41): p. 43117-25.
20. Schulthess, F.T., et al., *CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling.* Cell Metab, 2009. **9**(2): p. 125-39.
21. Kaiser, N., et al., *Monolayer culture of adult rat pancreatic islets on extracellular matrix: modulation of B-cell function by chronic exposure to high glucose.* Endocrinology, 1991. **129**(4): p. 2067-2076.
22. Maedler, K., et al., *Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function.* Diabetes, 2001. **50**(1): p. 69-76.
23. Ardestani, A., et al., *Neutralizing IL-1{beta} induces {beta}-cell survival by maintaining PDX1 nuclear localization.* J Biol Chem, 2011.
24. Le Bacquer, O., et al., *TCF7L2 splice variants have distinct effects on {beta}-cell turnover and function.* Hum Mol Genet, 2011. **20**(10): p. 1906-15.
25. Pham, N.A., et al., *Quantitative image analysis of immunohistochemical stains using a CMYK color model.* Diagn Pathol, 2007. **2**: p. 8.
26. Shu, L., et al., *Transcription factor 7-like 2 regulates beta-cell survival and function in human pancreatic islets.* Diabetes, 2008. **57**(3): p. 645-53.
27. Leng, S.X., et al., *ELISA and multiplex technologies for cytokine measurement in inflammation and aging research.* J Gerontol A Biol Sci Med Sci, 2008. **63**(8): p. 879-84.
28. Repnik, U., M. Knezevic, and M. Jeras, *Simple and cost-effective isolation of monocytes from buffy coats.* J Immunol Methods, 2003. **278**(1-2): p. 283-92.
29. Gammelsaeter, R., et al., *Complementary expression of SN1 and SAT2 in the islets of Langerhans suggests concerted action of glutamine transport in the regulation of insulin secretion.* Biochem Biophys Res Commun, 2009. **381**(3): p. 378-82.
30. Blixt, O., et al., *Sialoside specificity of the siglec family assessed using novel multivalent probes: identification of potent inhibitors of myelin-associated glycoprotein.* J Biol Chem, 2003. **278**(33): p. 31007-19.
31. Yamaji, T., et al., *A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9.* J Biol Chem, 2002. **277**(8): p. 6324-32.
32. Rapoport, E., et al., *Ganglioside binding pattern of CD33-related siglecs.* Bioorg Med Chem Lett, 2003. **13**(4): p. 675-8.

33. Bock, N. and S. Kelm, *Binding and inhibition assays for Siglecs.* Methods Mol Biol, 2006. **347**: p. 359-75.
34. Angata, T. and A. Varki, *Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily.* Glycobiology, 2000. **10**(4): p. 431-8.
35. Sasaki, C.Y., et al., *Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway.* J Biol Chem, 2005. **280**(41): p. 34538-47.
36. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.
37. Zhang, J.Q., et al., *Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes.* J Biol Chem, 2000. **275**(29): p. 22121-6.
38. Brunetta, E., et al., *The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia.* Blood, 2009. **114**(18): p. 3822-30.
39. Malisan, F. and R. Testi, *GD3 in cellular ageing and apoptosis.* Exp Gerontol, 2002. **37**(10-11): p. 1273-82.
40. De Maria, R., et al., *Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis.* Science, 1997. **277**(5332): p. 1652-5.
41. Malisan, F. and R. Testi, *GD3 ganglioside and apoptosis.* Biochim Biophys Acta, 2002. **1585**(2-3): p. 179-87.
42. Maedler, K., et al., *Glucose induces beta-cell apoptosis via upregulation of the Fas-receptor in human islets.* Diabetes, 2001. **50**: p. 1683-1690.
43. Loweth, A.C., et al., *Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation.* Diabetes, 1998. **47**(5): p. 727-732.
44. Prokazova, N.V. and L.D. Bergelson, *Gangliosides and atherosclerosis.* Lipids, 1994. **29**(1): p. 1-5.
45. Simon, B.M., et al., *Disialoganglioside GD3 is released by microglia and induces oligodendrocyte apoptosis.* Cell Death Differ, 2002. **9**(7): p. 758-67.
46. Bhunia, A.K., G. Schwarzmann, and S. Chatterjee, *GD3 recruits reactive oxygen species to induce cell proliferation and apoptosis in human aortic smooth muscle cells.* J Biol Chem, 2002. **277**(19): p. 16396-402.
47. Dotta, F., et al., *Pancreatic islet ganglioside expression in nonobese diabetic mice: comparison with C57BL/10 mice and changes after autoimmune beta-cell destruction.* Endocrinology, 1992. **130**(1): p. 37-42.
48. Miyagi, T., et al., *Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides.* J Biol Chem, 1999. **274**(8): p. 5004-11.
49. Sasaki, A., et al., *Overexpression of plasma membrane-associated sialidase attenuates insulin signaling in transgenic mice.* J Biol Chem, 2003. **278**(30): p. 27896-902.
50. Yoshizumi, S., et al., *Increased hepatic expression of ganglioside-specific sialidase, NEU3, improves insulin sensitivity and glucose tolerance in mice.* Metabolism, 2007. **56**(3): p. 420-9.
51. Kono, T., et al., *PPAR-gamma activation restores pancreatic islet SERCA2 levels and prevents beta-cell dysfunction under conditions of hyperglycemic and cytokine stress.* Mol Endocrinol, 2012. **26**(2): p. 257-71.

52. Moibi, J.A., et al., *Peroxisome proliferator-activated receptor-gamma regulates expression of PDX-1 and NKX6.1 in INS-1 cells.* Diabetes, 2007. **56**(1): p. 88-95.
53. Viardot, A., et al., *Obesity is associated with activated and insulin resistant immune cells.* Diabetes Metab Res Rev, 2012. **28**(5): p. 447-54.
54. Dasu, M.R. and I. Jialal, *Free fatty acids in the presence of high glucose amplify monocyte inflammation via Toll-like receptors.* Am J Physiol Endocrinol Metab, 2011. **300**(1): p. E145-54.
55. Smith, P.D., et al., *Intestinal macrophages and response to microbial encroachment.* Mucosal Immunol, 2011. **4**(1): p. 31-42.

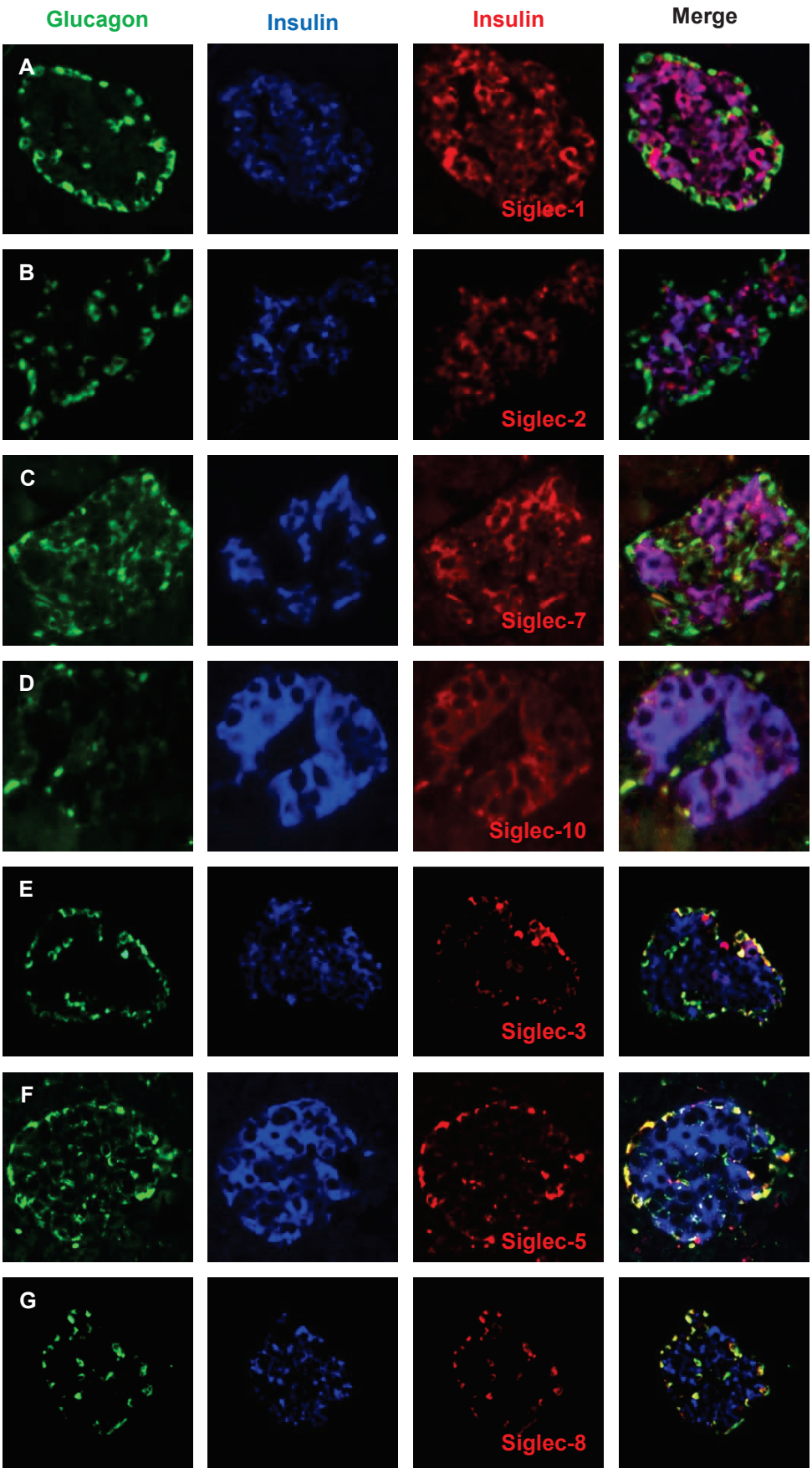
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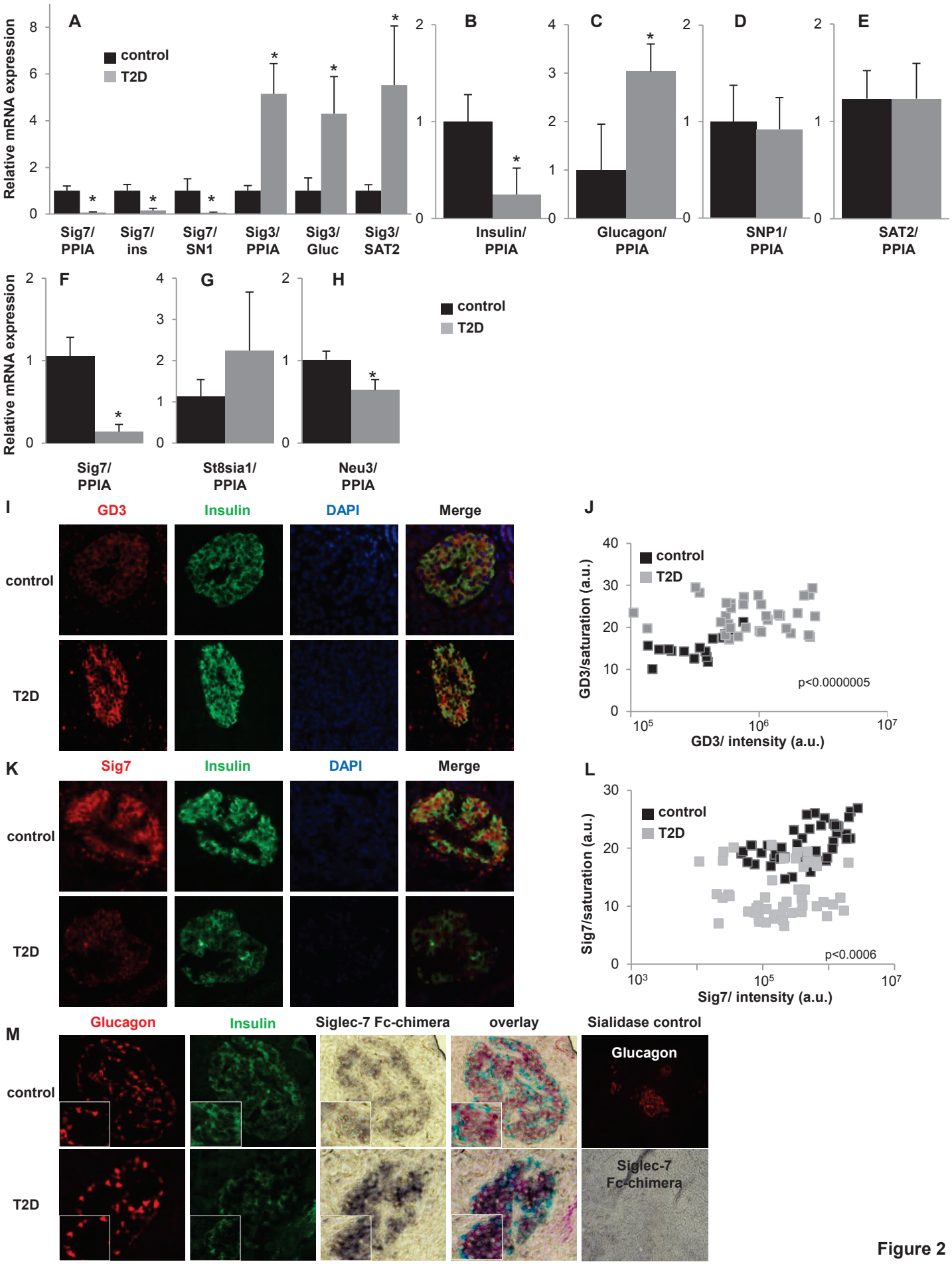
Figure 1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St8sia1/ PPIA
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Figure 2

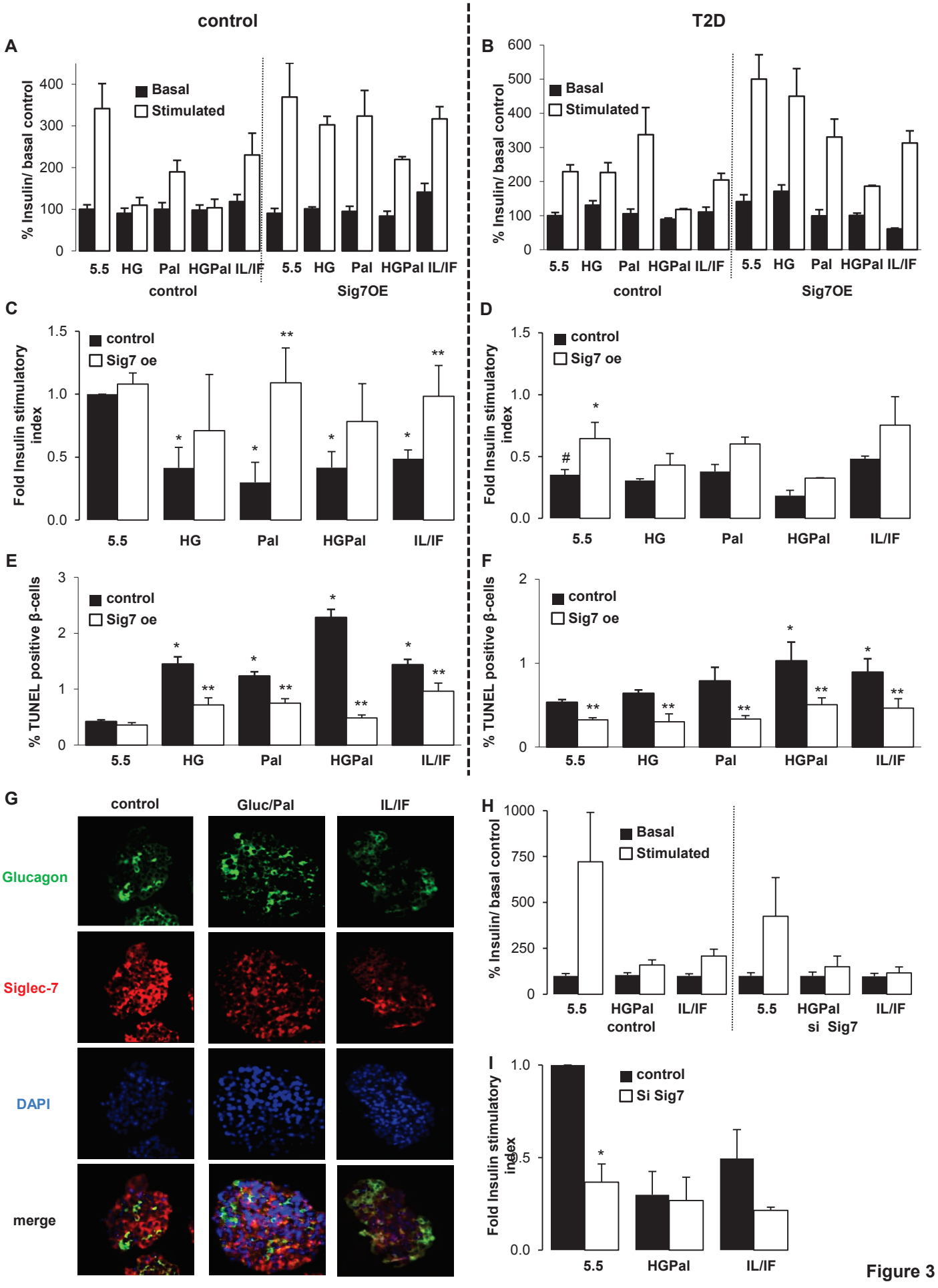
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Figure 3

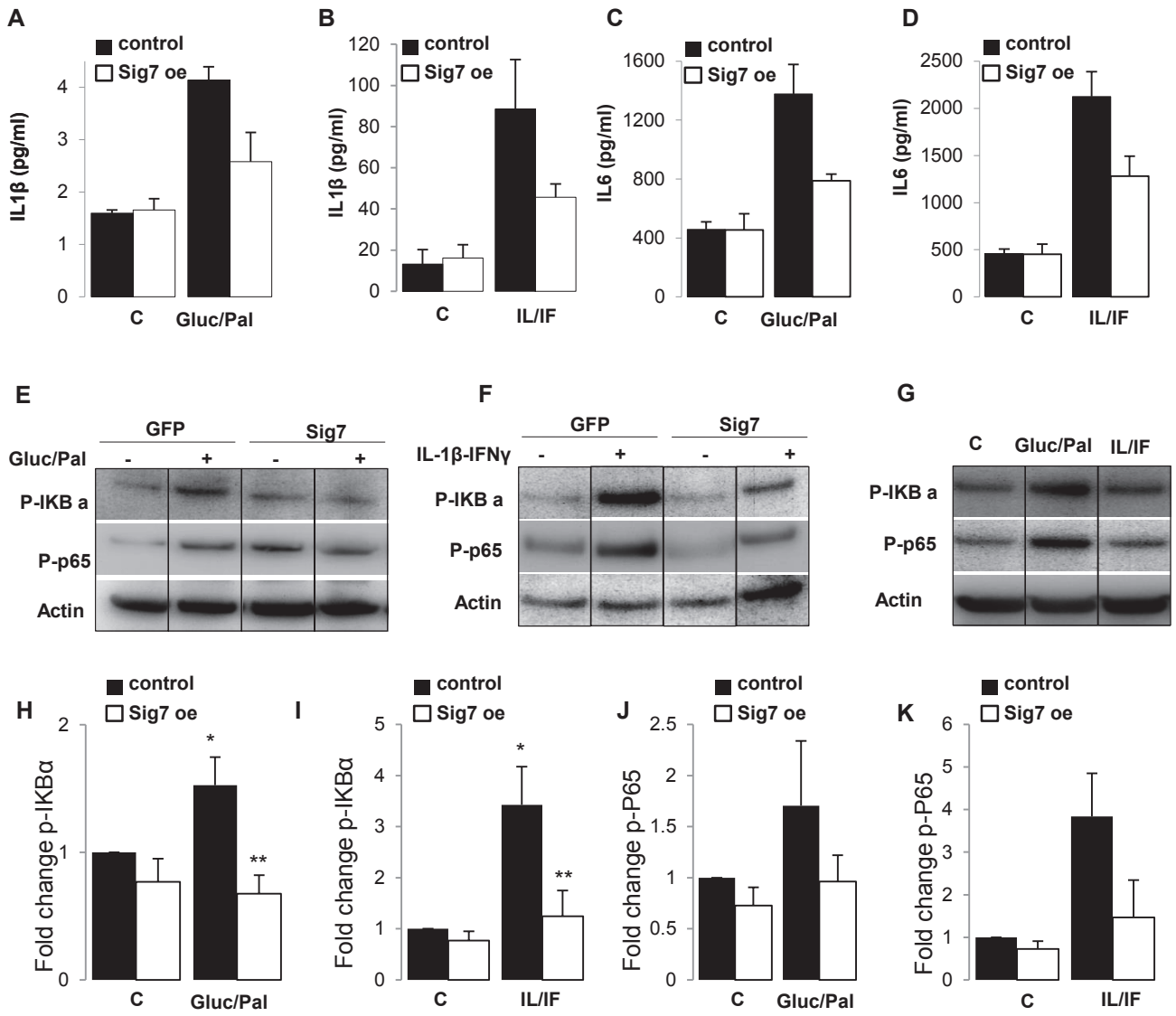
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Figure 4

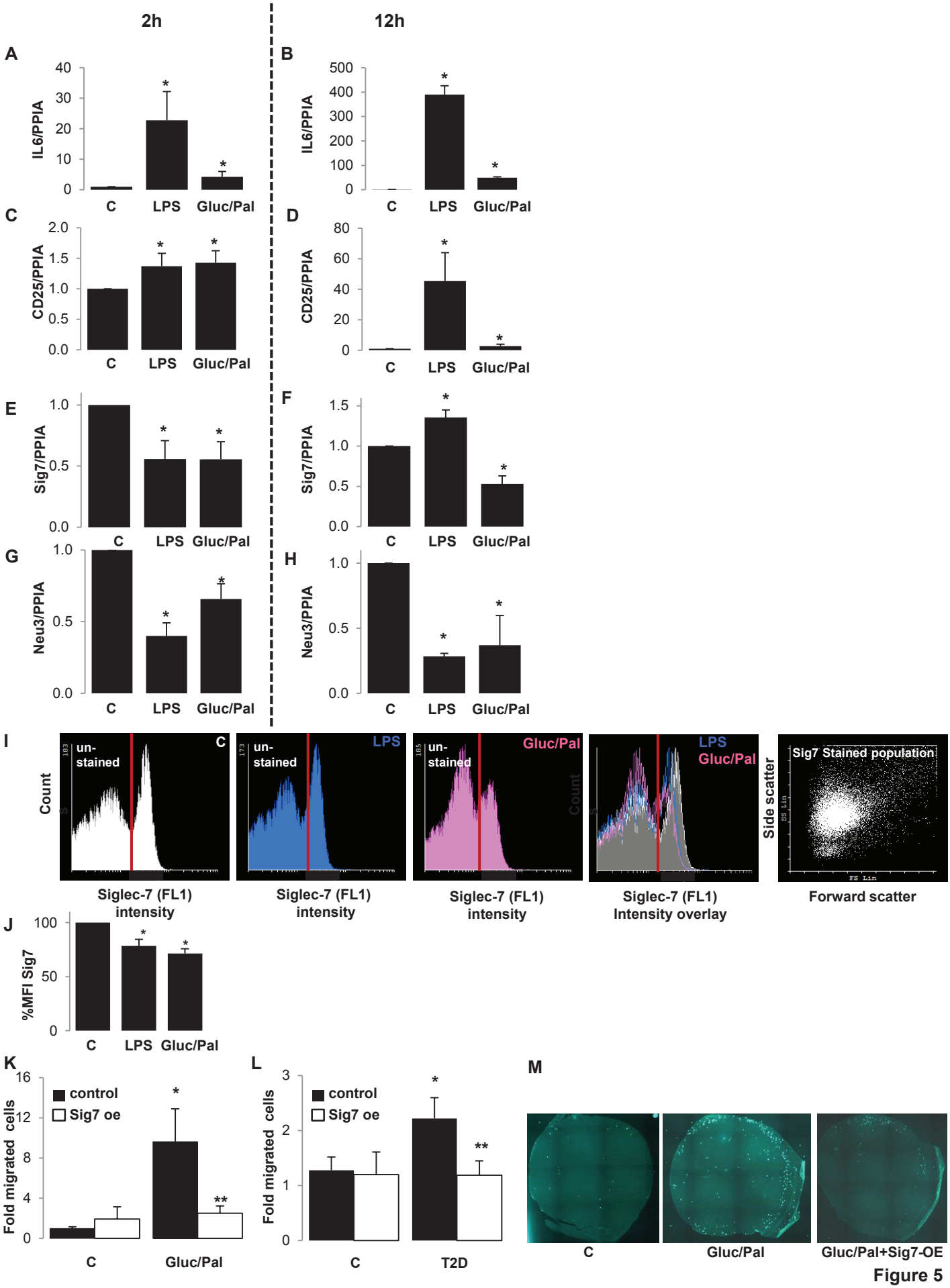
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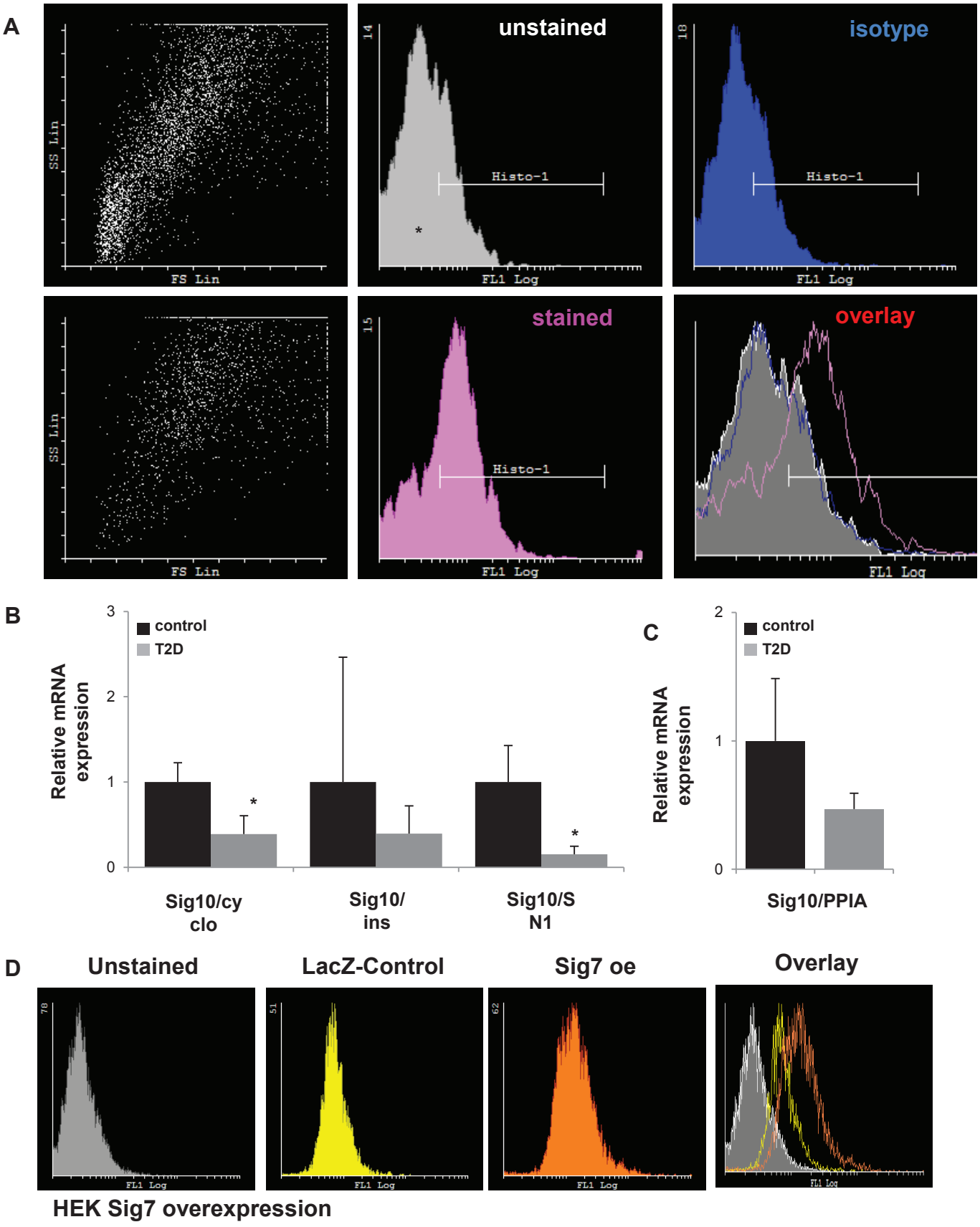
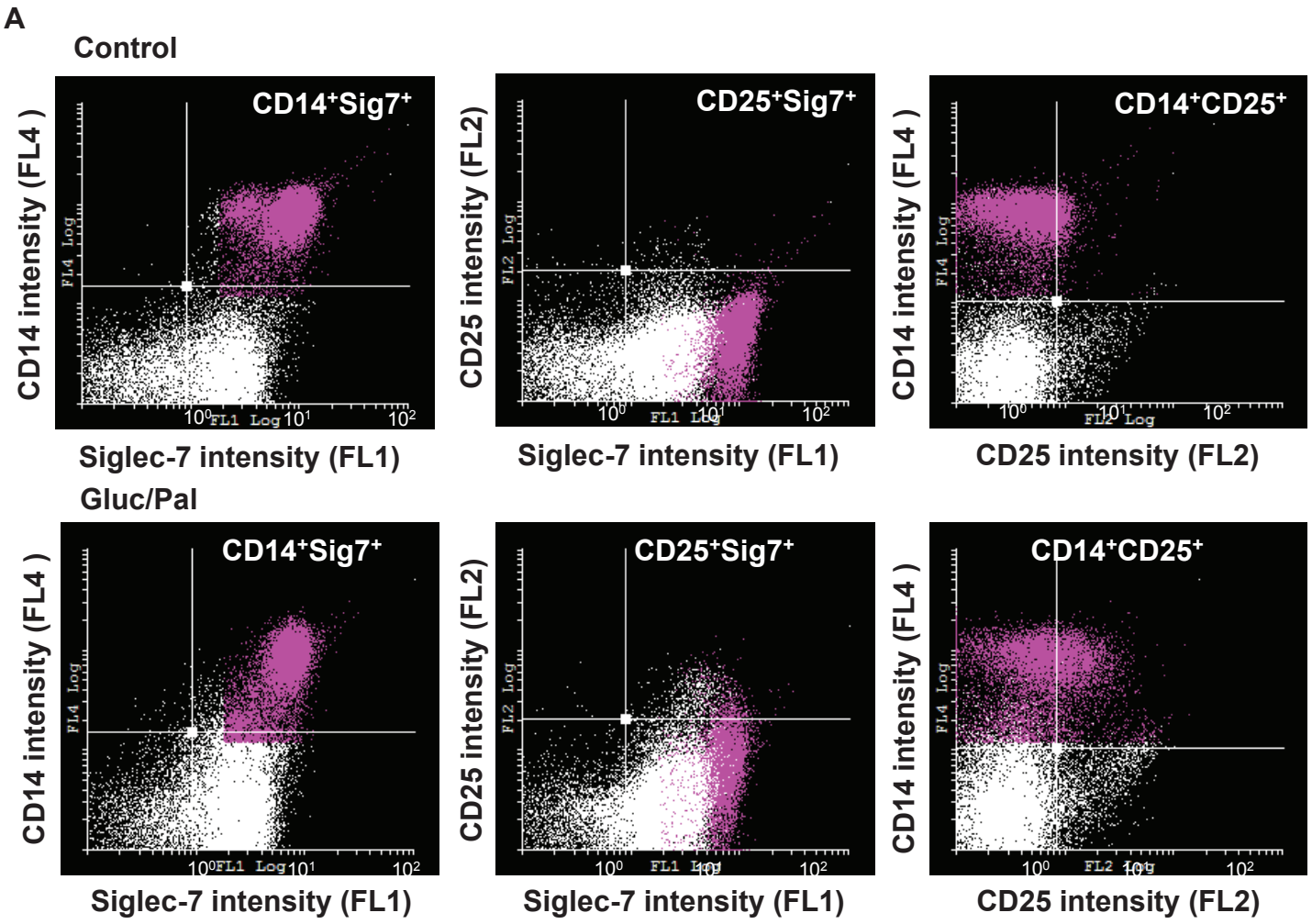
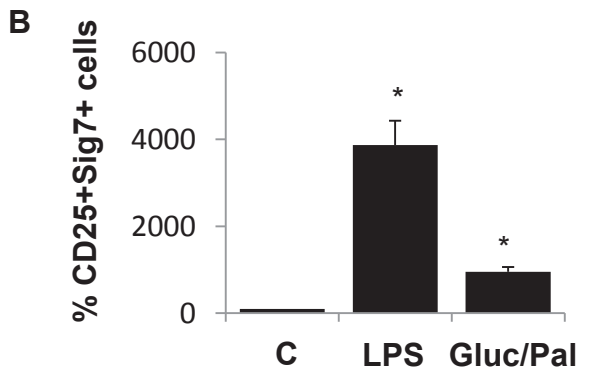
Figure 5

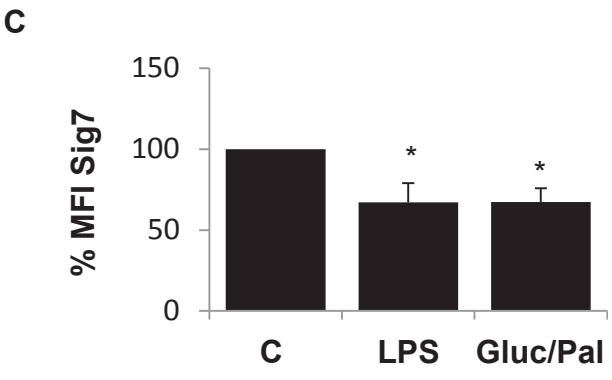
Supplementary figure 1

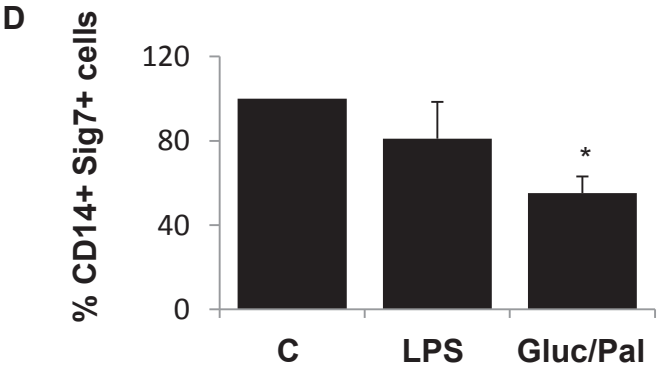


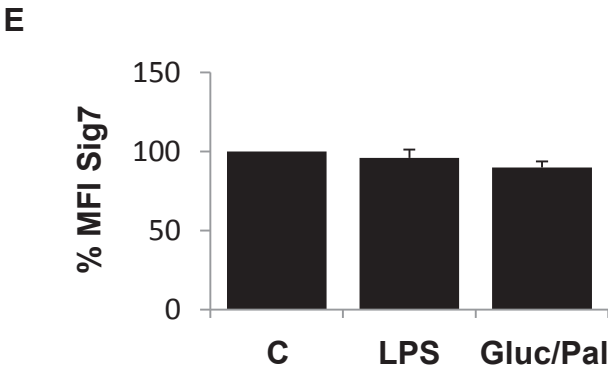
Supplementary figure 2



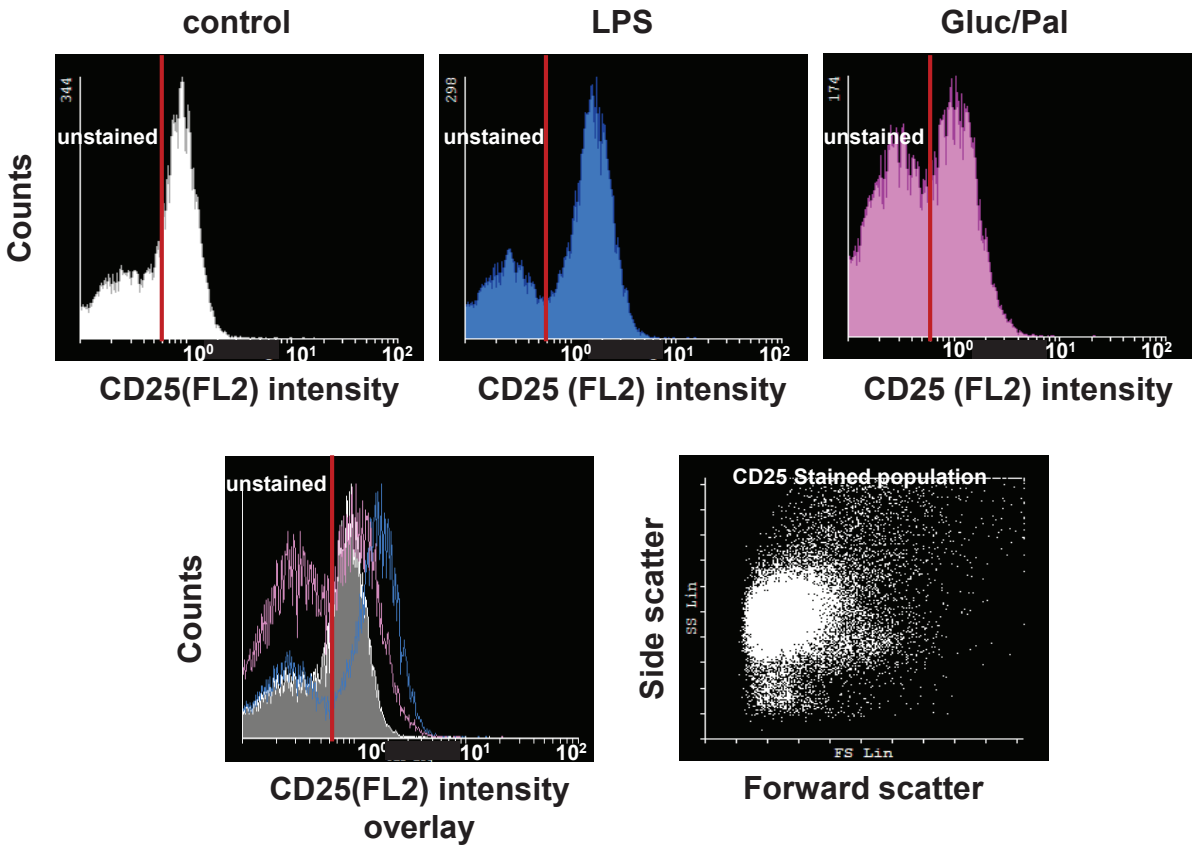




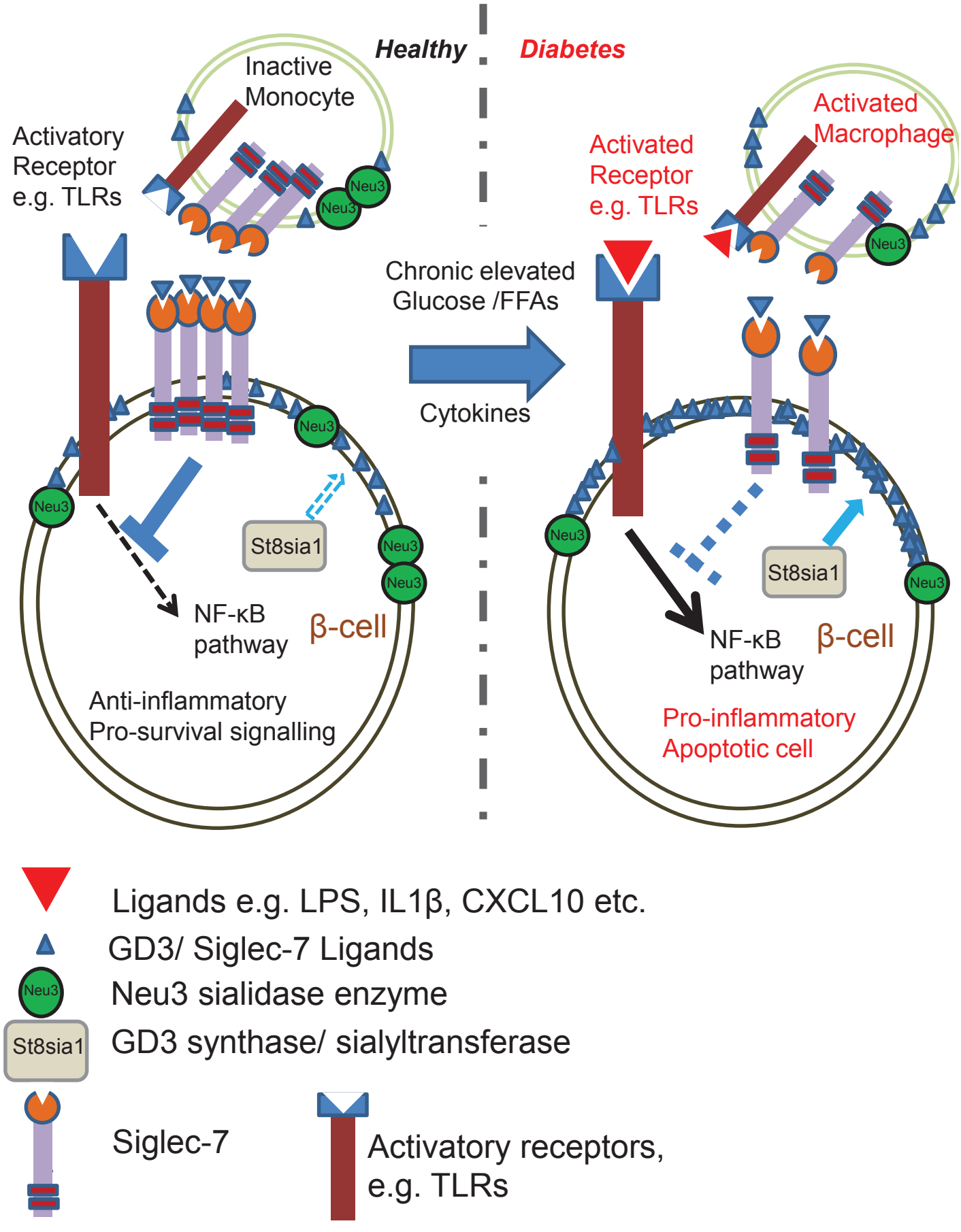




Supplementary figure 3



Supplementary figure 4



Discussion and Outlook

This thesis is a compilation of three mutually exclusive investigations which drive towards the sole aim of understanding the mechanisms and players in β-cell survival. The thread joining these investigations was to put forth therapeutic targets or strategies to rescue and preserve the β-cell mass and function in diabetes. The investigations could be broadly classified into three categories: preservation of β-cell mass, maintenance of β-cell function and amelioration of the pro-inflammatory environment in the islets. Thus, one study was aimed at identifying a molecule TOSO, which could act as a switch to drive the apoptosis of these cells into a proliferative mode, thus protecting the β-cell from Fas induced apoptosis. The second study looked deeper into effects of one of the existing medications: DPP4-inhibitors on β-cell function and survival. Third and the major research carried out during this thesis, was exploring β-cell function and survival under conditions of inflammation with a focus on the cell surface expression and signaling of siglecs. This section of the thesis will discuss each of these investigations in detail, always keeping in mind the basic aim of protecting the β-cells from deleterious events taking place in diabetes.

Target for induction of β-cell proliferation

TOSO switches Fas-induced β-cell apoptosis into proliferation [1].

The activation of inflammatory pathways has been discovered as a causal event for β-cell destruction in diabetes [2, 3], which is also mediated by TLR activation [4, 5]. Thereby, not only pro-inflammatory molecules are induced (e.g. IL-1β, Fas), but also the anti-inflammatory cytokine interleukin-1 receptor antagonist and the anti-apoptotic cFLIP are down-regulated in β-cells in diabetes [2, 6, 7]. Also, TOSO down-regulation was reported in response to TLR activation [8]. Thus, attempts to block Fas-induced apoptosis and the activation of pro-inflammatory cytokines could be a strategy to prevent diabetes. Here we show that down-regulation of another protective factor in T2D and its loss promotes β-cell destruction.

TOSO was first cloned and characterized in hematopoietic cells as a novel immunoglobulin domain-containing type I transmembrane protein, exhibiting potent pathway-specific anti-apoptotic effect [9]. The name TOSO was given after a Japanese liquor that is drunk on New Year's Day to celebrate long life and eternal youth [9], reflecting its pro-survival effect. TOSO inhibited apoptosis induced by Fas, TNFα, FADD, and PMA/ionomycin; but not staurosporine- or ceramide-

induced apoptosis. The mechanism of inhibition and its specificity were hypothesized to be due to inhibition of caspase-8 processing, through induction of cFLIP expression. The role of TOSO in maintaining cFLIP expression during Fas mediated apoptosis of lymphocytes and hepatocytes has been elucidated using $TOSO^{-/-}$ mice [10]. B-cells and thymocytes from these mice show increased sensitivity to Fas-triggered apoptosis, and these mice suffer greater mortality and exhibit exacerbated liver damage in response to Fas engagement *in vivo*. TOSO modulates Fas-mediated apoptosis by influencing the expression of c-FLIP and regulating the physical binding of caspase-8 to Fas receptor.

The anti-apoptotic function of TOSO depends on ubiquitination of an adaptor kinase, RIP1, and involves the recruitment of the death adaptor FADD to a TOSO/RIP1 protein complex. Upon activation by FasL and TNFα, TOSO promotes the activation of pro-survival signaling pathways and protects from liver damage [11]. TLR activation leads to decreased TOSO expression shown in leukemic B-cells [8].

In this study, I showed that TOSO expression correlated positively with β-cell proliferation; both the proliferation-inducing treatments, i.e., acute high glucose and low dose IL-1β positively regulated TOSO expression. On the other hand, pro-apoptotic stimuli, i.e., chronic elevated glucose and high dose of IL-1β almost depleted TOSO mRNA expression. Thus, depletion of TOSO coincides with activation of β-cell apoptosis under conditions of stress. TOSO, when over-expressed, not only rescued β-cells from apoptosis but also triggered proliferation. This pro-survival signaling has also been observed in TOSO over-expressing Jurkat cells in which CD95L- and TNFα-stimulation readily induced the activation of Erk1/2 [11]. While TNFα potentiates cell death, it is insufficient to cause apoptosis in β-cells [12]. Since TOSO promotes the TNFα -induced signaling cascade, it is possible that TNFα would induce β-cell apoptosis in the absence of TOSO, but there is no experimental proof for this hypothesis so far.

Intact Fas receptor signaling is also necessary for β-cell secretory function [13]. Hence, we investigated whether TOSO expression can also contribute to glucose stimulated insulin secretion. While TOSO over-expression was not effective at basal glucose levels, our results show that TOSO protected the inhibition of insulin secretion by elevated glucose and cytokines and restored β-cell function.

Mature human β-cells have only a very limited capacity to undergo proliferation [14]. To control and foster β-cell proliferation has become a long-term goal in β-cell research. With the goal to find a subpopulation of β-cells with a higher proliferative capacity, I over-expressed TOSO and investigated the nature of the induced proliferation by using

the technique of sequential thymidine analogue labelling, previously developed in mice by Teta *et al*. [15]. I optimized the method so that it could be used in a similar fashion *in vitro* in human islets, to detect multiple rounds of proliferation. TOSO over-expression induced an early round of proliferation in the isolated human islets, irrespective of sequence of analogue administration or time after transfection. However, only in a very limited number of islets (maximum 0.08 %), more than one round of proliferation was observed during the 6-day culture period. Such limited proliferation capacity is in confirmation with the results from mouse β-cells *in vivo*. In summary, TOSO is a novel anti-apoptotic protein, which interferes with the Fas triggered apoptosis by regulating FLIP and thereby initiating a pro-survival signaling cascade (see chapter 3.1.1 for our model). Thus, TOSO hints to be a promising therapeutic target to rescue β-cells from apoptosis induced by elevated glucose and cytokines and hence, intervening in the progression of diabetes. However, with the means of TOSO, we were unable to develop a β-cell, which would undergo multiple rounds of replication during culture.

Target for prevention of β-cell apoptosis and improvement of function

The DPP4- inhibitor Linagliptin improves β-cell function and survival by stabilising GLP-1 [16]

Orally administered DPP-4 inhibitors prevent the rapid cleavage of GLP-1 and thus increase levels of active GLP-1, resulting in increased insulin and reduced glucagon secretion, lowered glucose and reduced HbA1c levels by 0.5 – 1.0% [17]. Linagliptin improves glycemic control both as monotherapy [18] and in combination therapy, e.g., with metformin [19] or pioglitazone [20] and also improves insulin sensitivity [20]. DPP-4 inhibitors mimic many of the actions ascribed to GLP-1 including preservation of β-cell mass through stimulation of cell proliferation and inhibition of apoptosis in rodents [21-23].

Besides GIP and GLP-1, many gastrointestinal hormones, neuropeptides, cytokines, and chemokines are substrates for DPP-4 [24], including CXCL10 and SDF1α, both of which are involved in immune regulation, influence the inflammatory response and have been associated with the regulation of β-cell turnover in diabetes [5, 25]. Since cytokines and chemokines are crucial for the survival of pancreatic β-cells [26], it remained to be investigated whether DPP-4 inhibitors in culture directly affect β-cell survival and function. In the present study, the DPP-4 inhibitor linagliptin effectively restored proliferation, survival and β-cell function in a diabetic milieu. The

concentrations of 30 – 50 nM used in the study were higher than the average C_{max} plasma levels but could be also obtained in patients taking therapeutic doses of linagliptin [27]. At all concentrations up to 1 μM, which is 1000-fold the IC_{50} of the human enzyme, linagliptin did not affect β-cell survival at basal conditions in culture. Human β-cells in culture have only a very limited if any [28] capacity to proliferate. Here we found 0.4% proliferating β-cells under basal conditions when islets were plated on extracellular matrix coated dishes, and proliferation was even further decreased by diabetogenic conditions of chronically elevated glucose, free fatty acids, cytokines or H_2O_2. Linagliptin improved cell survival by decreasing apoptosis, and maintained cells at a steady state level, even under diabetogenic conditions.

The protective linagliptin effect seen in this study was mediated by stabilization of GLP-1 in the islet supernatants, where total as well as active GLP-1 levels were restored and accumulated during islet culture, providing a similar mechanism of the protective effect of DPP-4 inhibition on β-cell survival as *in vivo*.

Active GLP-1 can be produced from islets [29]. The β-cells express pro-hormone convertase PC1/3, which cleaves preproglucagon peptide to generate GLP-1. β-cells are an established islet source of secreted GLP-1 [30-33] and may provide a local protective paracrine effect for β-cell regeneration under diabetic conditions, especially during β-cell injury [25]. Sorted human β-cells also express PC1/3 but the GLP-1 content could not be detected and thus it was assumed that β-cells do not produce GLP-1 [34], although it was found to be secreted from the rodent β-cell line, INS-1E [35]. We found high levels of active GLP-1 (3 ng/ml from 50 islets over 4 days of culture) were secreted from human islets and accumulated only in the presence of a DPP-4 inhibitor, while high DPP-4 activity could only be measured in the absence of linagliptin.

GLP-1 is accumulated in the medium during the 4-day culture period and provides in turn a paracrine protective effect on the β-cell. Diabetogenic conditions like the cytokine cocktail IL-1β/IFNγ enhanced IL-6 production, which was counteracted by linagliptin, paralleled with increased GLP-1 in the medium. It can thus be hypothesized, that higher GLP-1 concentration in the culture medium in turn inhibits elevations in IL-6; this would provide a negative feedback loop to the IL-6 effect to induce GLP-1 in islets [30].

Hyperglycemia and a diabetic milieu decrease expression of β-cell GLP-1 receptors *in vivo* and *in vitro* [36-38]. In contrast, diabetogenic conditions significantly increased total GLP-1 release from cultured islets. This was also observed in islets from patients with

T2D [31] and from diabetic *Psammomys obesus [33]*, which show higher GLP-1 secretion than non-diabetic controls.

Slightly reduced GLP-1 secretion after a meal in patients with impaired oral glucose tolerance and more severely impaired GLP-1 secretion in type 2 diabetic patients was observed [39], although controversial results have been obtained in various studies [40]. Differences occur between tissue-specific GLP-1 levels and whether total or active GLP-1 was measured [41]. It is possible, that reduced GLP-1 in T2D patients is a result of elevated plasma DPP-4 activity [42], which can be induced by chronic hyperglycemia and is paralleled with reductions of active GLP-1 [43].

Linagliptin strongly reduced DPP-4 activity in the human islet supernatant and stimulate GLP-1 stability. Active DPP-4 rapidly degrades SDF-1α(1-68) to inactive SDF-1α(3-68) [44] and thus, more active SDF-1α is available upon DPP-4 inhibition. In turn SDF-1α acts like a cytokine and induces its own production [25]. This explains the increased SDF-1α mRNA levels upon linagliptin treatment, found in the present study.

The regulation of DPP-4 in diabetes has not been fully clarified, both increased [42, 43] and decreased [45] DPP-4 activity has been reported in patients with diabetes. DPP-4 release from adipose tissue is higher in obese individuals and correlates with parameters of the metabolic syndrome, where DPP-4 directly impairs insulin signaling in fat and muscle cells [46]. Elevated glucose exposure leads to enhanced DPP-4 activity and mRNA expression in endothelial cells [47]. Also in human islets, we detected elevated DPP-4 mRNA in response to elevated glucose and palmitate, while linagliptin inhibited an induction. One could speculate that in islets under diabetogenic conditions, there is more DPP-4 produced, which inactivates GLP-1 and thus it cannot fulfil its functions.

Recent human studies investigating the long-term treatment with vildagliptin on β-cell function were somehow disappointing, because following wash out periods the observed β-cell sparing effects disappeared, thus questioning the disease modifying potential of this drug [48]. However, it remains to be elucidated whether this is caused by a limited tissue penetration *in vivo* or limited availability of the drugs in islets. In summary, I show that linagliptin has a protective effect on β-cell turnover and function, which is mediated through stabilized GLP-1 and an anti-inflammatory/anti-oxidative stress pathway.

Target for amelioration of inflammation-induced β-cell death

Siglec-7 inhibits pro-inflammatory signaling in stressed β-cells, and improves their survival and function

The present study investigated the role of sialic acid-binding immunoglobulin-like lectins (Siglecs), a novel, still-expanding family of cell adhesion molecules, in islet inflammation observed in T2D. Here, we show for the first time, that some of the Siglecs, previously known to be majorly expressed in the haematopoietic cells, are also expressed, in a cell type specific manner, in the endocrine pancreas. Of the evolutionarily evolving CD-33 related siglecs which were investigated, Siglec-7 and Siglec-10 were expressed solely in the β-cells, whereas siglecs 3, 5 and 8 were expressed only in the α-cells. Although, Siglec-7 shares around 84 % sequence homology with Siglec-9 [49], its presence was undetectable in the endocrine pancreas as assessed by real-time PCR and immunohistochemical analyses. The non-hematopoietic siglecs, Siglec-4 (myelin associated) and Siglec-6 (placental) were also not detected in the islets. Out of the siglecs which are evolutionarily conserved within species, siglecs 1 and 2 are expressed in the β-cells. Siglecs being cell adhesion molecules, this differential expression could imply their involvement in intra-islet cellular cross talk.

After having confirmed their presence in islets, we checked whether expressions of any of these siglecs were affected in diabetic islets. The β-cell specific siglecs 7 and 10 were markedly down-regulated in pancreas of individuals with T2D. In contrast, Siglec-3 was significantly up-regulated in α-cells in T2D.

Owing to the significant, drastic regulation of Siglec-7 in diabetes, we focused our research on its role in the β-cells. Previously, it has been shown by Brunetta E. *et al*. that a marked decrease in the surface expression of Siglec-7 is the foremost marker of the aberrant NK-cell dysregulation in patients with chronic HIV-1 viremia [50]. We saw strong correlation between incidence of T2D and down-regulation of Siglec-7 in the β-cells. Also, in vitro down-regulation of Siglec-7 in the β-cells was sufficient to impair the β-cell function. Thus, we hypothesized that the loss of Siglec-7 in the β-cells contributed to their dysfunction and apoptosis. Indeed, restoring surface Siglec-7 expression protected the β-cells from deleterious effects of a diabetic milieu. Siglec-7 could not only restore the glucose stimulated insulin secretion under these conditions, but it also inhibited β-cell apoptosis. This rescue of function and survival was also evident in *in vitro* studies of freshly isolated T2D islets. Hence, we could conclude that loss of Siglec-

7 in the β-cells contributed to their destruction observed in T2D. Nevertheless, one needs to take into consideration the fact that over-expression in islets in the present study could occur in all its different cell types. Further experiments using an insulin promoter driven plasmid might be useful in confirming the β-cell specific role of Siglec-7.Another fact that needs to be focused on is that, Siglec-3 also has cytoplasmic ITIMs, but its functional significance in α-cells needs to be further investigated. Also, it is one C2-set domain shorter than Siglec-7, and might vary its interactions within the islets.

Siglec-7 has an unusual binding preference for α-2,8-linked disialic acids and weaker interactions with branched α-2,6 sialyl residues [51]. Subsequently, we immunohistochemically investigated the presence of these binding partners in the human pancreas using Siglec-7 Fc-chimeras, and found them to be expressed in islets. Interaction partners were present in both α- and β-cells, indicating the possibility of intra-islet *trans* interactions of Siglec-7 with its ligands on both these cell types.

An endogenous ligand of Siglec-7, ganglioside GD3, was strongly upregulated in diabetes. The disialoganglioside GD3 is an acidic glycosphingolipid, generated downstream of the ceramide-driven ganglioside biosynthesis, by sialylation of its immediate precursor GM3 by the GD3 synthase (α- 2,8-sialyltransferase or ST8Sia I or SAT II). In freshly isolated islets obtained from autopsy of patients with T2D, we could detect a tendency of increase in ST8SiaI expression, which supported our observed upregulation of GD3 in diabetic individuals. As early as in 1979, GD3 has been first described as a tumor-associated antigen, with its overexpression observed in different melanomas [52]. More than two decades later, its involvement in the Fas and ceramide mediated apoptosis in haematopoietic cells was reported [53]. GD3 directly targets mitochondria and disrupts mitochondrial trans-membrane potential [53], leading to the release of apoptotic factors such as ROS, cytochrome c, AIF and caspase-9 [54]. Its induced expression in diabetes, thus, hints not only towards feedback up-regulation of ligand upon loss of Siglec-7 expression, but also gives an indirect proof of activation of the pro-apoptotic signaling like that of the Fas receptor, previously reported by us in the context of glucotoxicity in the islets [7]. Increased levels of GD3 in serum have also been implicated in inflammatory processes such as atherosclerosis [55]. GD3 acts as an extracellular messenger of cell death in the lipopolysaccharide triggered inflammation in brain, wherein the microglial cells are activated and secrete GD3 leading to apoptosis of oligodendrocytes [56]. Islet inflammation in T2D has been elucidated by induction of cytokine and chemokine secretion upon chronic exposure to

elevated glucose and free fatty acids [57]. We have previously shown induced IL1β expression by the β-cell as a result of chronic elevated glucose concentrations resulting in β-cell apoptosis [2]. Parallels can be drawn between the cytokine IL1β and GD3, as both at low concentrations, stimulate cell proliferation while at high concentration triggers apoptosis [2, 58]. Hence, increased GD3 expression reiterates the pro-apoptotic inflamed state of islets in T2D.

Together with the increased GD3 synthase, we also observed decreased Neu3 expression in islets isolated from individuals with T2D. Neu3 is the membrane-associated sialidase which is specific for gangliosides [59]. It has been reported that mice over-expressing NEU3 mainly in muscles develop severe insulin-resistant diabetes [60]. However, hepatic NEU3 overexpression paradoxically improves insulin sensitivity and glucose tolerance through modification of ganglioside composition and Peroxisome Proliferator-activated Receptor gamma (PPAR-γ) signaling [61]. In islets, PPAR-γ activation restores β-cell function under conditions of hyperglycemia and cytokine stress [62] and its agonist are used in pharmacological treatment of T2D. In line with these observations, decreased Neu3 expression in T2D islets can lead to reduced PPAR- γ signaling, and hence might contribute to the β-cell dysfunction under diabetogenic conditions. In addition to this, Neu3 can cleave off the surface sialic acid residues, which can unmask Siglec-7 and induce its inhibitory signaling cascade [63]. This unmasking is also diminished in T2D, as observed by significantly lower levels of Neu3 and increased ligand expression in diabetic islets.

Along with the observed induced pro-apoptotic glycosylation machinery, loss of the inhibitory receptor Siglec-7 further accentuates loss of β-cell function and survival. We investigated underlying mechanisms of the protective effect of Siglec-7 by performing western blot analysis of transfected and treated islets, and checked for activation of the classical pathway. Chronic exposure of islets to diabetic stimuli led to increased phosphorylation of IKBα, marking it for proteosomal degradation. Once free, p65/p50 NF-κB heterodimer could translocate to the nucleus where it would be phosphorylated, thus enhancing its transcriptional activity. Diabetic milieu could also induce this p65 phosphorylation. Siglec-7 over-expression blocked this signaling cascade by inhibiting phosphorylation of IκBα and p65. The consequent secretion of cytokines after the NF-κB activation was investigated, which revealed decreased expression and secretion of cytokines Il1b and IL-6 upon Siglec-7 restoration in islets treated with a diabetic milieu of elevated glucose and palmitate as well as mixture of cytokines IL1β/IFNγ. This

demonstrates the immuno-suppresive effect of Siglec-7, which can protect the β-cells from cytokine-mediated apoptosis.

Chronic activation of the innate immune system is observed in both T1D and T2D. Metabolic changes in glucose homeostasis have been shown to affect the inflammatory state of the immune system It has been shown in the context of T1D, soluble form Interleukin receptor 7-α is increased, and it has been attributed to effect of hyperglycemia on the T-cells [64]. We wanted to check the regulation of Siglec-7 in the immune cells. As Siglec-7 is endogenously expressed mainly by natural killer cells and monocytes, we checked for its regulation upon acute and chronic treatment of freshly isolated peripheral blood monocytes (PBMCs) with elevated glucose and palmitate as well as cytokine mixture. Siglec-7 down-regulation was seen to be a hallmark of the activated monocytes. Acute exposure to LPS as well as elevated glucose and palmitate was sufficient to inhibit Siglec-7 expression. In spite of that, chronic exposure to LPS led to restoration of the messenger RNA, but maintained reduced cell surface expression of Siglec-7. On the other hand, glucose and palmitate chronically kept both the mRNA and protein levels down, proving their chronic effect on the activation of the immune cells. In both acute and chronic treatment, cytokine IL-6 was induced. CD25, an atypical marker for activated macrophages [65] was induced after chronic treatment with the diabetic milieu, reiterating the activation of these cells. The down-regulation of Siglec-7 in activated PBMCs goes in hand with the decreased expression in islets under conditions of inflammation, which highlights its anti-inflammatory role in both these cell types. Ultimately, we investigated the effect of restoration of siglecs in the islets on the actual infiltration of the immune cells. The increased number of macrophages per islet observed *in vivo* in diabetes [66] could, for the first time, be confirmed *in vitro* using a leukocyte migration assay, wherein we could quantify the glucolipotoxicity induced migration of immune cells towards the islets. Maintaining Siglec-7 expression in these stressed islets could inhibit the recruitment and migration of the immune cells. Hence, Siglec-7 expression in islets is essential for maintaining an anti-inflammatory environment in islets which prevents subsequent immune system activation.

Summarizing the findings, we detected the presence of a novel family of cell adhesion molecules- Siglecs, expressed in the endocrine cells of the pancreas. One of its β-cell specific members, Siglec-7 was lost in diabetes. Restoration of Siglec-7 in these cells could protect them from the harmful effects of diabetic milieu, and help preserve β-cell function and survival under these conditions by inhibition of pro-inflammatory cytokine

secretion by suppressing NF-κB activity. Not only was this immune-modulatory function evident in the cytokine profile of the β-cells, but also the PBMCs showed loss of Siglec-7 expression upon activation.

As a therapeutic agent, Siglec-7 is a very lucrative target as it can rescue both the activated immune system as well as the inflamed islet. Restoration of Siglec-7 signaling both in islets and immune cells may halt the progression of the pro-inflammatory state in diabetes. Crosslinking the siglecs using synthetic ligands may be able to restore the inhibitory signals. The binding of synthetic sialic acid derivative with high affinity to Siglec-2 have been shown to modulate the inhibitory activity of its ITIM [67]. Development of similar high affinity ligands for Siglec-7 can be a strategy for treatment of diabetes (Figure 1).

Taken together, Siglec-7 plays a substantial role in the maintenance of immune-suppressive anti-inflammatory microenvironment, which is lost in diabetes, and may contribute to the manifestation and progression of this metabolic syndrome. Thus, preserving Siglec-7 expression and function on β-cells as well as immune cells may be a novel therapeutic strategy which could help target both, the sensitisation and pro-inflammatory activation of the immune system as well as the islets, thereby being beneficial to effectively halt the progress of islet destruction in diabetes.

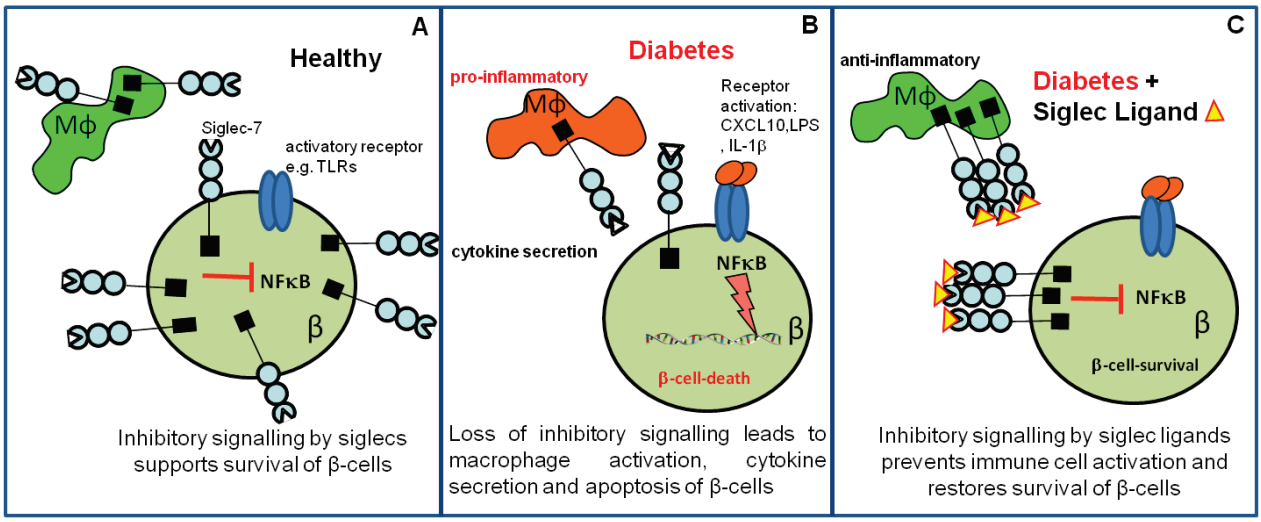


Figure 1.Siglec-7 in diabetes: summary and outlook (adapted from [68]). (A) In healthy individuals, inhibitory signaling by Siglec-7 supports the survival of β-cells. **(B)** However, in diabetes, Siglec-7 expression is lost on β-cells as well on macrophages; inhibition of NFκB is lost, leading to macrophage activation, cytokine secretion and apoptosis of β-cells. **(C)** Restoration of inhibitory signals may be brought about by ligands which would cluster Siglec-7, preventing immune cell activation and β-cell death.

Zooming out to view the bigger picture

At first glance, this thesis might come across as a collage of three different investigations, without focus on any one particular aspect in islet research. Without refuting such claims, I would like to point out the fine points of similarities between the three projects, which clarified their purpose during my thesis.

Diabetes manifests from multi-organ failure, at the crux of which lies the loss of β-cell function and survival. In spite of that, there is a lack of treatments targeted to maintain endogenous β-cell mass. Current therapies aim at reducing postprandial hyperglycemia. Pharmacological interventions currently in use are insulin secretagogues (sulfonylureas and meglitinides), which bind to β-cells to induce insulin secretion, insulin sensitizers (thiazolidinediones), which improve insulin response, Biguanides like metformin, which act primarily to reduce hepatic gluconeogenesis, and α-glucosidase and SGLT2 inhibitors, which reduce carbohydrate reabsorption from the intestine and kidney [69-72], but none of the current treatments provides a cure for diabetes, namely restoration of the β-cells, only a cure of the symptoms.

β-cell death is a complex phenomenon, wherein multiple triggers act via simultaneous activation of several pathways. Therefore, it is essential to not only focus on specific stimuli and its consequences, but also the general tissue specific impact of a mixture of stimuli which mimic the scenario *in vivo*.

This thesis brings forth two different molecules which may be pursued further as potential targets for restoring β-cell function and mass in T2D. Even though the modes of action of both TOSO and Siglec-7 are diverse, their β-cell protective function needs to be further investigated in order to understand the full extent of their role in these systems. Additionally, this thesis contributes to the mechanistic understanding of the DPP-4 inhibitor linagliptin. Taken together, my dissertation is a part of a body of research which aims to prevent and/or cure diabetes by directly solving the central failure in overt diabetes: loss of β-cell function and mass.

References

1. Dharmadhikari, G., et al., *The Fas apoptotic inhibitory protein TOSO induces proliferation in human beta cells.* Molecular Metabolism, 2012. **1**.
2. Maedler, K., et al., *Glucose-induced beta-cell production of interleukin-1beta contributes to glucotoxicity in human pancreatic islets.* J.Clin.Invest, 2002. **110**: p. 851-860.
3. Donath, M.Y., et al., *Cytokine production by islets in health and diabetes: cellular origin, regulation and function.* Trends Endocrinol Metab, 2010. **21**(5): p. 261-7.
4. Ehses, J.A., et al., *Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet.* Diabetologia, 2010. **53**(8): p. 1795-806.
5. Schulthess, F.T., et al., *CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling.* Cell Metab, 2009. **9**(2): p. 125-39.
6. Maedler, K., et al., *Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets.* Proc Natl Acad Sci U S A, 2004. **101**(21): p. 8138-43.
7. Maedler, K., et al., *Glucose induces beta-cell apoptosis via upregulation of the Fas-receptor in human islets.* Diabetes, 2001. **50**: p. 1683-1690.
8. Vire, B., A. David, and A. Wiestner, *TOSO, the Fcmicro receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation.* J Immunol, 2011. **187**(8): p. 4040-50.
9. Hitoshi, Y., et al., *Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells.* Immunity, 1998. **8**(4): p. 461-71.
10. Huo, J., et al., *Genetic deletion of faim reveals its role in modulating c-FLIP expression during CD95-mediated apoptosis of lymphocytes and hepatocytes.* Cell Death Differ, 2009. **16**(7): p. 1062-70.
11. Nguyen, X.H., et al., *Toso regulates the balance between apoptotic and nonapoptotic death receptor signaling by facilitating RIP1 ubiquitination.* Blood, 2011. **118**(3): p. 598-608.
12. Omori, K., et al., *mRNA of the pro-apoptotic gene BBC3 serves as a molecular marker for TNF-alpha-induced islet damage in humans.* Diabetologia, 2011. **54**(8): p. 2056-66.
13. Schumann, D.M., et al., *The Fas pathway is involved in pancreatic {beta} cell secretory function.* Proc Natl Acad Sci U S A, 2007. **104**: p. 2861-2866.
14. Rutti, S., et al., *In vitro proliferation of adult human Beta-cells.* PLoS One, 2012. **7**(4): p. e35801.
15. Teta, M., et al., *Very slow turnover of beta-cells in aged adult mice.* Diabetes, 2005. **54**(9): p. 2557-67.
16. Shah, P., et al., *The DPP-4 inhibitor linagliptin restores beta-cell function & survival in human isolated islets through GLP-1 stabilization.* J Clin Endocrinol Metab, 2013.
17. Drucker, D.J. and M.A. Nauck, *The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes.* Lancet, 2006. **368**(9548): p. 1696-705.
18. Del Prato, S., et al., *Effect of linagliptin monotherapy on glycaemic control and markers of beta-cell function in patients with inadequately controlled type 2 diabetes: a randomized controlled trial.* Diabetes Obes Metab, 2011. **13**(3): p. 258-67.

19. Taskinen, M.R., et al., *Safety and efficacy of linagliptin as add-on therapy to metformin in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled study.* Diabetes Obes Metab, 2011. **13**(1): p. 65-74.
20. Gomis, R., et al., *Efficacy and safety of initial combination therapy with linagliptin and pioglitazone in patients with inadequately controlled type 2 diabetes: a randomized, double-blind, placebo-controlled study.* Diabetes Obes Metab, 2011. **13**(7): p. 653-61.
21. Pospisilik, J.A., et al., *Dipeptidyl peptidase IV inhibitor treatment stimulates beta-cell survival and islet neogenesis in streptozotocin-induced diabetic rats.* Diabetes, 2003. **52**(3): p. 741-50.
22. Cho, J.M., et al., *A novel dipeptidyl peptidase IV inhibitor DA-1229 ameliorates streptozotocin-induced diabetes by increasing beta-cell replication and neogenesis.* Diabetes Res Clin Pract, 2011. **91**(1): p. 72-9.
23. Pospisilik, J.A., et al., *Long-term treatment with dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulin sensitivity in the VDF Zucker rat: a euglycemic-hyperinsulinemic clamp study.* Diabetes, 2002. **51**(9): p. 2677-83.
24. De Meester, I., et al., *Natural substrates of dipeptidyl peptidase IV.* Adv Exp Med Biol, 2000. **477**: p. 67-87.
25. Liu, Z., et al., *Stromal cell-derived factor-1 (SDF-1)/chemokine (C-X-C motif) receptor 4 (CXCR4) axis activation induces intra-islet glucagon-like peptide-1 (GLP-1) production and enhances beta cell survival.* Diabetologia, 2011. **54**(8): p. 2067-76.
26. Maedler, K., et al., *Interleukin-targeted therapy for metabolic syndrome and type 2 diabetes.* Handb Exp Pharmacol, 2011(203): p. 257-78.
27. Graefe-Mody, U., S. Retlich, and C. Friedrich, *Clinical pharmacokinetics and pharmacodynamics of linagliptin.* Clin Pharmacokinet, 2012. **51**(7): p. 411-27.
28. Portha, B., C. Tourrel-Cuzin, and J. Movassat, *Activation of the GLP-1 receptor signaling pathway: a relevant strategy to repair a deficient beta-cell mass.* Exp Diabetes Res, 2011. **2011**: p. 376509.
29. D'aleo, V., et al., *The direct effects of DPP-4 inhibition on isolated human islets include protection from glucotoxicity.* Diabetologia, 2009. **52**: p. S35-S36.
30. Ellingsgaard, H., et al., *Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells.* Nat Med, 2011. **17**(11): p. 1481-9.
31. Marchetti, P., et al., *A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets.* Diabetologia, 2012. **55**(12): p. 3262-3272.
32. Whalley, N.M., et al., *Processing of proglucagon to GLP-1 in pancreatic alpha-cells: is this a paracrine mechanism enabling GLP-1 to act on beta-cells?* J Endocrinol, 2011. **211**(1): p. 99-106.
33. Hansen, A.M., et al., *Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in Psammomys obesus--an adaptive response to hyperglycaemia?* Diabetologia, 2011. **54**(6): p. 1379-87.
34. Kirkpatrick, C.L., et al., *Type 2 diabetes susceptibility gene expression in normal or diabetic sorted human alpha and beta cells: correlations with age or BMI of islet donors.* PLoS One, 2010. **5**(6): p. e11053.
35. Chepurny, O.G. and G.G. Holz, *Over-expression of the glucagon-like peptide-1 receptor on INS-1 cells confers autocrine stimulation of insulin gene promoter activity: a strategy for production of pancreatic beta-cell lines for use in transplantation.* Cell Tissue Res, 2002. **307**(2): p. 191-201.

36. Cheong, Y.H., et al., *Glucose exposure pattern determines glucagon-like peptide 1 receptor expression and signaling through endoplasmic reticulum stress in rat insulinoma cells.* Biochem Biophys Res Commun, 2011. **414**(1): p. 220-5.
37. Shu, L., et al., *Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with downregulation of GIP- and GLP-1 receptors and impaired beta-cell function.* Hum Mol Genet, 2009. **18**(13): p. 2388-99.
38. Xu, G., et al., *Downregulation of GLP-1 and GIP receptor expression by hyperglycemia: possible contribution to impaired incretin effects in diabetes.* Diabetes, 2007. **56**(6): p. 1551-8.
39. Nauck, M.A., et al., *Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: what is up, what is down?* Diabetologia, 2011. **54**(1): p. 10-8.
40. Meier, J.J. and M.A. Nauck, *Is the diminished incretin effect in type 2 diabetes just an epi-phenomenon of impaired beta-cell function?* Diabetes, 2010. **59**(5): p. 1117-25.
41. Mussig, K., et al., *Genetic variants affecting incretin sensitivity and incretin secretion.* Diabetologia, 2010. **53**(11): p. 2289-97.
42. Ryskjaer, J., et al., *Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbAlc levels, but is not acutely affected by food intake.* Eur J Endocrinol, 2006. **155**(3): p. 485-93.
43. Mannucci, E., et al., *Hyperglycaemia increases dipeptidyl peptidase IV activity in diabetes mellitus.* Diabetologia, 2005. **48**(6): p. 1168-72.
44. Hocher, B., C. Reichetzeder, and M.L. Alter, *Renal and Cardiac Effects of DPP-4 Inhibitors - from Preclinical Development to Clinical Research.* Kidney Blood Press Res, 2012. **36**(1): p. 65-84.
45. Meneilly, G.S., et al., *Effect of ageing and diabetes on glucose-dependent insulinotropic polypeptide and dipeptidyl peptidase IV responses to oral glucose.* Diabet Med, 2000. **17**(5): p. 346-50.
46. Lamers, D., et al., *Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome.* Diabetes, 2011. **60**(7): p. 1917-25.
47. Pala, L., et al., *Dipeptidyl peptidase-IV expression and activity in human glomerular endothelial cells.* Biochem Biophys Res Commun, 2003. **310**(1): p. 28-31.
48. Foley, J.E., et al., *Beta cell function following 1 year vildagliptin or placebo treatment and after 12 week washout in drug-naive patients with type 2 diabetes and mild hyperglycaemia: a randomised controlled trial.* Diabetologia, 2011. **54**(8): p. 1985-91.
49. Zhang, J.Q., et al., *Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes.* J Biol Chem, 2000. **275**(29): p. 22121-6.
50. Brunetta, E., et al., *The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia.* Blood, 2009. **114**(18): p. 3822-30.
51. Yamaji, T., et al., *A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9.* J Biol Chem, 2002. **277**(8): p. 6324-32.
52. Portoukalian, J., G. Zwingelstein, and J.F. Dore, *Lipid composition of human malignant melanoma tumors at various levels of malignant growth.* Eur J Biochem, 1979. **94**(1): p. 19-23.

53. De Maria, R., et al., *Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis.* Science, 1997. **277**(5332): p. 1652-5.
54. Malisan, F. and R. Testi, *GD3 ganglioside and apoptosis.* Biochim Biophys Acta, 2002. **1585**(2-3): p. 179-87.
55. Prokazova, N.V. and L.D. Bergelson, *Gangliosides and atherosclerosis.* Lipids, 1994. **29**(1): p. 1-5.
56. Simon, B.M., et al., *Disialoganglioside GD3 is released by microglia and induces oligodendrocyte apoptosis.* Cell Death Differ, 2002. **9**(7): p. 758-67.
57. Donath, M.Y., et al., *Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity.* Cell Metab, 2013. **17**(6): p. 860-72.
58. Bhunia, A.K., G. Schwarzmann, and S. Chatterjee, *GD3 recruits reactive oxygen species to induce cell proliferation and apoptosis in human aortic smooth muscle cells.* J Biol Chem, 2002. **277**(19): p. 16396-402.
59. Miyagi, T., et al., *Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides.* J Biol Chem, 1999. **274**(8): p. 5004-11.
60. Sasaki, A., et al., *Overexpression of plasma membrane-associated sialidase attenuates insulin signaling in transgenic mice.* J Biol Chem, 2003. **278**(30): p. 27896-902.
61. Yoshizumi, S., et al., *Increased hepatic expression of ganglioside-specific sialidase, NEU3, improves insulin sensitivity and glucose tolerance in mice.* Metabolism, 2007. **56**(3): p. 420-9.
62. Kono, T., et al., *PPAR-gamma activation restores pancreatic islet SERCA2 levels and prevents beta-cell dysfunction under conditions of hyperglycemic and cytokine stress.* Mol Endocrinol, 2012. **26**(2): p. 257-71.
63. Nicoll, G., et al., *Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms.* Eur J Immunol, 2003. **33**(6): p. 1642-8.
64. Monti, P., et al., *Concentration and activity of the soluble form of the interleukin-7 receptor alpha in type 1 diabetes identifies an interplay between hyperglycemia and immune function.* Diabetes, 2013. **62**(7): p. 2500-8.
65. Smith, P.D., et al., *Intestinal macrophages and response to microbial encroachment.* Mucosal Immunol, 2011. **4**(1): p. 31-42.
66. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.
67. Kelm, S., et al., *The ligand-binding domain of CD22 is needed for inhibition of the B cell receptor signal, as demonstrated by a novel human CD22-specific inhibitor compound.* J Exp Med, 2002. **195**(9): p. 1207-13.
68. Kathrin Maedler, P.C., Beat Ernst and Soerge Kelm, *Ligands of Siglecs for treatment of T1D*. 2013, JDRF.
69. Yki-Jarvinen, H., *Thiazolidinediones.* N Engl J Med, 2004. **351**(11): p. 1106-18.
70. Ahren, B., *Are sulfonylureas less desirable than DPP-4 inhibitors as add-on to metformin in the treatment of type 2 diabetes?* Curr Diab Rep, 2011. **11**(2): p. 83-90.
71. Israili, Z.H., *Advances in the treatment of type 2 diabetes mellitus.* Am J Ther, 2011. **18**(2): p. 117-52.
72. Seino, S., et al., *Treating diabetes today: a matter of selectivity of sulphonylureas.* Diabetes Obes Metab, 2012. **14 Suppl 1**: p. 9-13.

Appendix

1. IL1β targeted therapy for type 2 diabetes

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Diabetes: perspectives in drug therapy

Contribution: wrote chapter f IL1β in brain

2. Neutralizing Interleukin-1β (IL-1β) Induces β-Cell Survival by maintaining PDX1 Protein Nuclear Localization in Journal of Biological Chemistry (JBC)

Contribution. Assessment of β-cell survival in mouse tissue sections

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Interleukin-1 beta targeted therapy for type 2 diabetes

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Since having been cloned in 1984, IL-1β has been the subject of over 22,000 citations in Pubmed, among them over 800 reviews. This is because of its numerous effects. IL-1β is a regulator of the body's inflammatory response and is produced after infection, injury, and antigenic challenge. It plays a role in various diseases, including autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases and type 1 diabetes, as well as in diseases associated with metabolic syndrome such as atherosclerosis, chronic heart failure and type 2 diabetes. Macrophage are the primary source of IL-1, but epidermal, epithelial, lymphoid and vascular tissues also synthesize IL-1. IL-1β production and secretion have also been reported from pancreatic islets. Insulin-producing β-cells within pancreatic islets are specifically prone to IL-β-induced destruction and loss of function. Macrophage-derived IL-1β production in insulin-sensitive organs, leads to progression of inflammation and induction of insulin resistance in obesity. We summarize the mechanisms involved in inflammation and specifically the IL-1β signals that lead to the progression of insulin resistance and diabetes. We highlight recent clinical studies and experiments in animals and isolated islets using IL-1β as a potential target for the therapy of type 2 diabetes.

Keywords: diabetes, IL-1Ra, IL-1β, β-cell

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1. Introduction

During the 25 years after IL-1β was cloned in the lab of Charles Dinarello [1], production and secretion of IL-1β have been linked not only to various autoimmune and auto-inflammatory diseases, but also to metabolic dysregulation [2]. Meanwhile, 11 ligands and 10 receptors of the IL-1 family have been discovered. The pro-inflammatory and agonistic ligands are IL-1α, IL-1β, IL-18, family of interleukin 1 (FIL-1)ε, IL-1H2, IL-1ε, IL-33 and the anti-inflammatory ligands IL-1Ra, FIL1δ, IL-1H4, IL-1Hy2 [2]. IL-1α, IL-1β and IL-1Ra bind to IL-1R1, IL-1β and the IL-1β precursor bind to IL-1R2, IL-33 binds to IL-1R4, IL-18 and IL-1H4 to IL-1R5, FIL-1ε, IL-1H2, IL-1ε to IL-1R6. IL-1R8, 9 and toll-interleukin 1 receptor 8 (TIR8) remain orphan receptors and IL-1RAcP and IL-18β are co-receptors [3]. In the pancreatic islet, IL-1R1 is present on the β-cells [4] and not the α-cells [5] and thus the β-cells are a target for IL-1α, IL-1β and IL-1Ra. This review summarizes signaling pathways of IL-1β which lead to impaired insulin secretion and insulin action. Clearly, other cytokines and chemokines are involved in the inflammatory responses; however, we focus here on the possibility of blocking only IL-1β as a target for improving glycemia in type 2 diabetes mellitus (T2DM).

2. IL-1β signaling in the β-cell

Soon after the cloning of IL-1β, Mandrup-Poulsen and colleagues observed that IL-1β impairs β-cell function [6,7]. In addition to impaired insulin secretion,

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IL-1β was found to induce β-cell death, which was potentiated by the cytokines IFNγ and TNFα [8,9]. Blocking IL-1β with specific IL-1β-neutralizing antibodies conferred protection from the cytotoxic effects induced by activated-mononuclear-cell-conditioned medium [10], indicating that IL-1β plays an important role in the molecular mechanisms underlying autoimmune β-cell destruction.

Since then, IL-1β signaling and the underlying mechanisms of IL-1β-induced β-cell destruction have been investigated. Importantly, IL-1β induces its own expression and the expression of other cytokines, for example IL-2, -3, -6 and interferons [11]. In turn, cells that produce IL-1β also respond to IL-1β [12]. IL-1β initiates signal transduction by binding to IL-1R1 on the β-cell. This leads to docking of the interleukin 1 receptor accessory protein (IL-1RAcP) to the IL-1–IL-1R1 complex, which is followed by recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MyD88). interleukin-1 receptor-associated kinase (IRAK)-4, Tollip and IRAK-1 are then recruited, allowing IRAK-1 to activate TNF-receptor-associated factor 6 (TRAF6), which in turn triggers activation of TGF-beta activated kinase 1 (TAK1). TAK1 is able to stimulate two main pathways; the inhibitor of NF-κB kinase (IKK)–NF-κB pathway, and the MAPK/stress-activated protein kinase (MAPK/SAPK) pathway [13]. In addition to TAK1, MAPK/extracellular-signal regulated kinase(ERK) kinase kinase 1 (MEKK1) seems to participate in the activation of both NFκB and SAPK in β-cells [14]. Phosphorylation of I-κB, a cytosolic inhibitor of NF-κB, by IKK leads to I-κB degradation and NF-κB translocation to the nucleus, thus regulating the transcription of many target genes, such as iNOS expression and production of NO, a toxic reactive radical. Consistently interfering with NF-κB activation decreases IL-1β-induced β-cell death [15,16].

IL-1β can also activate protein kinase Cδ, which leads to β-cell apoptosis presumably through iNOS expression [17,18]. Notably, IL-1β induces Fas expression on β-cells [19,20], increasing their sensitivity to FasL and accelerating apoptosis via cleavage of downstream caspases (**Figure 1** and reviewed in [21]). A distal consequence of IL-1β signaling in β-cells is the induction of endoplasmic reticulum (ER) stress. IL-1β depletes ER Ca^{2+}, leading to ER stress and induction of several ER stress markers including CCAAT/enhancer-binding protein homologous protein (CHOP). The induction of ER stress by IL-1β can be prevented by inhibition of iNOS, suggesting that NO mediates ER stress [22]. This is consistent with the notion that a chemical NO donor causes ER Ca^{2+} depletion and ER stress [23]. What is currently unclear is the importance of ER stress in IL-1β-induced β-cell impairment. Studies addressing the role of ER stress-induced CHOP so far indicates that ER stress and CHOP do not contribute to cytokine-induced β-cell death [24]. Thus, while there is little doubt that ER stress is induced in β-cells by IL-1β, it is uncertain whether ER stress contributes to apoptosis or whether it may simply be a secondary effect and thus only plays a minor role, if any, in IL-1β-mediated apoptosis.

The MAPK/SAPK pathways consist of ERK1/2, p38 and JNK1/2, all of which are activated by IL-1β in β-cells [25,26]. Using both pharmacological and molecular inhibitor approaches, NF-κB, ERK1/2, p38 and JNK1/2 have been demonstrated to be involved in IL-1β-induced β-cell apoptosis [25,27-30].

Another target of IL-1β signaling in β-cells is the survival kinase pathway PI3K–Akt. IL-1β reduces both PI3K [31] and Akt [32] activation. Since Akt is a negative regulator of JNK/SAPK in β-cells [33], reduced Akt signaling may allow increased and sustained pro-apoptotic JNK activation.

In general, signal transduction initiated by a ligand binding to membrane receptors leads to activation or induction of a negative feedback mechanisms to ensure only transient signaling. This is also true for signal transduction evoked by pro-inflammatory cytokines such as IL-1β. IL-1β induces expression of suppressor of cytokine signaling (SOCS)-3 in β-cells [31,34]. SOCS-3 is a member of a family of proteins that function to terminate cytokine signaling thereby constituting a negative feedback loop [35]. Although IL-1β induces SOCS-3 expression in β-cells, this induction seems to be insufficient to completely terminate IL-1β signal transduction, since prolonged NF-κB and MAPK/SAPK signaling is observed in β-cells exposed to IL-1β [25,33,36]. Putatively, either the amount of SOCS-3 induced by IL-1β in β-cells is too low to effectively block signaling or the kinetics of SOCS-3 induction by IL-1β is abnormally slow in β-cells. In any case, forced SOCS-3 overexpression effectively inhibits IL-1β signaling at the level of TRAF6, leading to dampening of both the NF-κB and MAPK/SAPK pathways thus protecting against apoptosis [13,37]. Interestingly, IL-1β induced endogenous SOCS-3 targets insulin signaling in the β-cell by associating with the insulin receptor (IR) thereby preventing activation of insulin receptor substrate (IRS) and PI3K [31]. By this mechanism, SOCS-3 induction is likely to contribute to IL-1β-induced desensitization of insulin signaling which is important for optimal β-cell function. One may speculate whether IL-1β-induced SOCS-3 expression is preferentially directed towards IR signals while leaving the IL-1β signaling cascade unaffected. The IL-1β signaling pathways are summarized in **Figure 1**.

3. IL-1β secretion

The primary sources of IL-1β are blood monocytes, tissue macrophages, and dendritic cells. B lymphocytes and NK cells also produce IL-1β [2]. The release of the leaderless cytokine, IL-1β, cannot be initiated through the Golgi apparatus. IL-1β precursor accumulates in the cytosol, and is processed by caspase-1 (also named Interleukin converting enzyme (ICE)) into the mature secreted IL-1β. This is triggered by ATP activating the $P2X_7$ receptor, which forms a pore in response to ligand stimulation and regulates cell permeability and cytokine release [38].

Resident islet macrophages are fundamental in the development of autoimmune diabetes [39,40] and it has

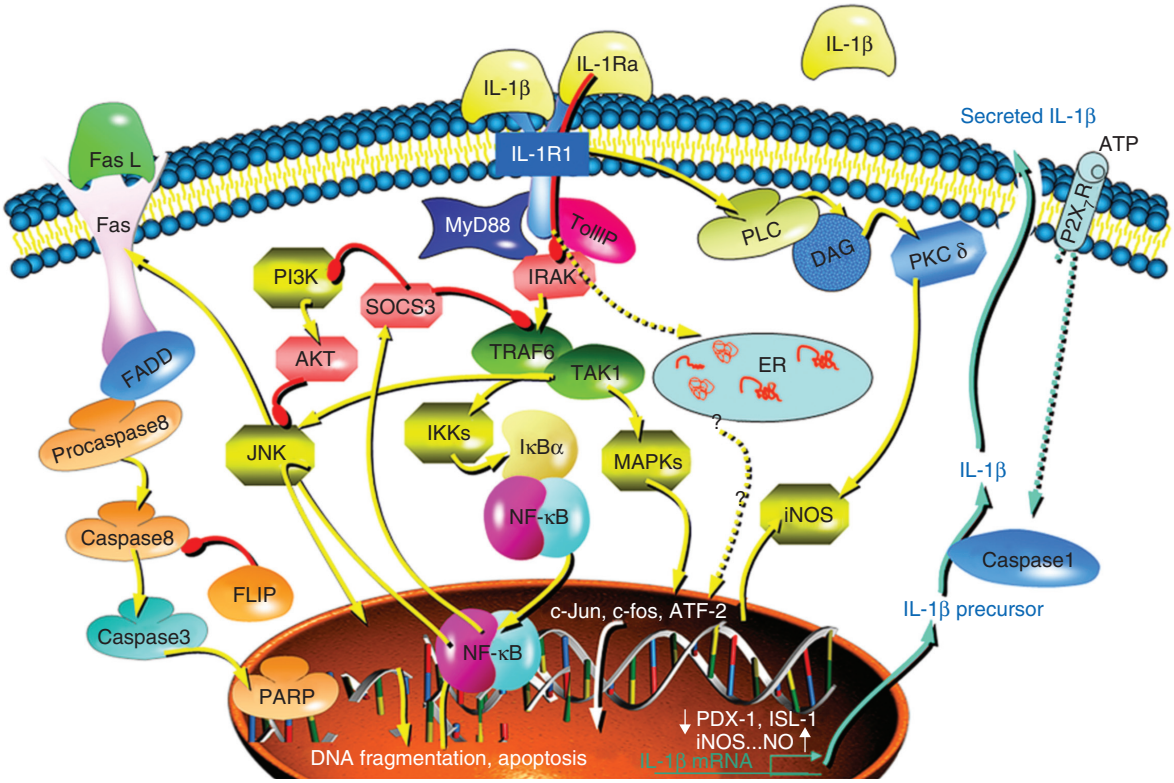

Figure 1. Mechanisms of IL-1β signaling in the β-cell. Details are described in the text.
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been postulated that IL-1β secreted from such intra-islet macrophages results in β-cell destruction [39]. More recent studies show that the β-cells themselves are able to secrete IL-1β which is induced by double stranded RNA, a mechanism by which viral infection may mediate β-cell damage [41] or by elevated glucose concentrations [42,43]. Upregulation of the Fas receptor plays a central role in the mediation of β-cell death [44,45]. IL-1β rapidly induces Fas upregulation, whereas glucose only induces Fas in chronic conditions [46].

In two animal models, *Psammomys obesus* and Goto-Kakizaki (GK) rat, it has been observed that pancreatic β-cells express IL-1β under hyperglycemic conditions [42,47]. In *Psammomys obesus*, normalizing hyperglycemia with phlorizin, an inhibitor of the renal tubular glucose re-uptake, inhibited intra-islet IL-1β expression [42]. In contrast, Jorns *et al.* found no IL-1β expression within the islets [48]. IL-1β production by islet cells was confirmed in several studies [49,43]. While glucose-induced IL-1β mRNA production was not found in human islets that had been preincubated in suspension for 3 – 5 days, Boni-Schnetzler *et al.* showed that glucose response in islets is negatively correlated with basal IL-1β expression levels [43]. These studies show that IL-β may mediate β-cell destruction also in T2DM (reviewed in [44]). It is tempting to suggest IL-1β as a target for the treatment of diabetes. However, whether changes in circulating cytokines are physiologically relevant in the face of locally produced inflammatory mediators remains unknown.

4. Blocking IL-1β signals protects the β-cell from IL-1β- and glucose-induced cell death

As described above, IL-1β has been shown to impair insulin release, to induce Fas expression thus enabling Fas-triggered apoptosis in rodent and human islets [15,50-59], and to share similarities with glucose-induced apoptosis. In parallel with the essential role of glucose in mediating insulin secretion and proliferation, a low concentration of IL-1β also stimulates insulin release and proliferation in rat and human islets [60-64]. The beneficial IL-1β effects seem to be partly mediated by the increased secretion of the naturally occurring anti-inflammatory cytokine and antagonist of IL-1α and IL-1β, the interleukin-1-receptor antagonist. IL-1Ra was discovered in 1987 [65-77]. Four forms of IL-1Ra have been described, three of them are intracellular proteins (icIL-1Ra I, II and III) and one is secreted (sIL-1Ra) [68]. Similar to IL-1β, IL-1Ra binds to type 1 and 2 IL-1 receptors but lacks a second binding domain. Therefore, IL-1Ra does not recruit the IL-1 receptor accessory protein, the second component of the receptor complex.

Endogenous production and secretion of IL-1Ra limits inflammation and tissue damage [2]. *In vivo*, exogenous

IL-1Ra counteracts low dose streptozotocin-induced diabetes [69], autoimmune diabetes [70] and promotes graft survival [71-83] and islet survival after transplantation [74].

We have recently shown that IL-1Ra is secreted from the β-cell and expressed in β-cell granules [75]. IL-1Ra protects cultured human islets from the deleterious effects of glucose [42] as well as IL-1β [57,71,72,76,77]. Inhibition of IL-1Ra with small interfering RNAs or long term treatment with leptin lead to β-cell apoptosis and impaired function, which may provide a further link between obesity and diabetes.

The definite secretion and regulation mechanisms of IL-1Ra are unknown. Like IL-1β, IL-1Ra may also be secreted by a leaderless pathway via activation of the $P2X_7$ receptor [78,79]. In pancreatic islets from obese individuals $P2X_7$ receptors are highly expressed and these receptors were almost undetectable in T2DM [79]. In accordance with the $P2X_7$ receptor expression levels, increased IL-1Ra serum levels correlate with obesity and insulin resistance [80-83], but IL-1Ra is decreased in T2DM [84]. Recent results from the Whitehall Study show that IL-1Ra levels are increased before the onset of T2DM [85], which are consistent with findings in mice fed with a high fat/high sucrose diet (HFD). IL-1Ra levels were increased after 4 and 8 weeks of diet together with an increase in β-cell mass and body weight. Serum concentrations of IL-1Ra are influenced by adipose tissue, which is a major source of IL-1Ra [86]. After 16 weeks, when the HFD-fed mice displayed glucose intolerance and β-cell apoptosis, IL-1Ra levels were lower than in the normal-diet-fed mice. Mice deficient for the $P2X_7$-receptor, were unable to increase β-cell mass in compensation in response to the HFD feeding and had no adaptive increase in IL-1Ra levels [79].

The increased IL-1Ra could be an attempt by the body to counteract the deleterious effects of IL-1β and to preserve β-cell survival, insulin secretion and insulin sensitivity. It has been suggested that IL-1Ra has an additional metabolic effect, which leads to insulin resistance. However, when we treated mice daily for 12 weeks with IL-1Ra we did not observe changes in insulin sensitivity at any time point [87].

Whether serum IL-1Ra levels would explain the progression of diabetes in obese individuals and whether serum IL-1Ra affects IL-1Ra expression in the β-cell is not known. We suggest that a decreased β-cell IL-1Ra expression could trigger the progression from obesity to diabetes and high IL-1Ra expression could protect the β-cell and enable it to adapt to conditions of higher insulin demand, this is illustrated in **Figure 2**.

5. IL-1β links obesity and diabetes

Chronic sub-clinical inflammation is present in obesity, insulin resistance and T2DM. The diseases related to metabolic syndrome are characterized by abnormal cytokine production, including elevated circulating IL-1β, increased acute-phase proteins, e.g., CRP [88] and activation of inflammatory signaling pathways [89].

Proinflammatory cytokines can cause insulin resistance in adipose tissue, skeletal muscle and liver by inhibiting insulin signal transduction. The sources of cytokines in insulin-resistant states are the insulin target tissues themselves, primarily fat and liver, but to a larger extent the activated tissue-resident macrophages [90].

While macrophage infiltration in adipose and brain tissue has been shown in many studies [85], increased islet macrophage infiltration has only recently been observed in pancreatic sections from patients with T2DM [86], and in T2DM animal models, such as the GK rat [91], the HFD and *db*/*db* mouse [86], and the hyperglycemic Cohen diabetic rat [92]. While IL-1β signals induce destruction and impaired insulin secretion in the β-cells, insulin signaling is disturbed in the insulin target tissues (**Figure 3**).

Insulin receptor signaling is complex. To summarize shortly, signaling downstream of the insulin receptor involves phosphorylation of IRS1/2 and the activation of the PI3K–AKT pathway (responsible for insulin action on glucose uptake) and the Ras–MAPK pathway (responsible for suppression of gluconeogenesis, reviewed in [93]). Due to inflammation, IRS1 can be alternatively phosphorylated on serine 307, which leads to downstream activation of the NF-κB pathway, phosphorylation of Jnk1, activation of the JNK/activator protein 1 (AP-1) pathway and thus disturbed insulin signaling. Furthermore, IL-1β induces SOCS signaling which leads to degradation of IRS proteins [94].

6. IL-1β in adipocytes

Infiltration of macrophages in adipose tissue is tightly correlated with obesity in mice and humans [95,96]. Important modulators of inflammation are the adipocytokines, such as leptin, resistin and adiponectin which play a central role in the regulation of insulin resistance and β-cell function [97,98].

In obesity, not only circulating free fatty acids (FFA), and lipids, but also leptin and resistin are increased, whereas adiponectin, which is known to prevent inflammation [98] and which is negatively correlated with insulin resistance, is decreased [99]. Leptin has been shown to exert pro- as well as anti-inflammatory properties, probably dependent on its dose and exposure time. While *in vivo*, leptin overexpression normalizes glycemia in the diabetic NOD mice as well as in streptozotocin- and alloxan-induced diabetes [100], chronic leptin incubation *in vitro* leads to impaired β-cell function and survival [75,101,102]. Leptin has been shown to manipulate levels of IL-1β and IL-1Ra. While acutely, leptin induces IL-1Ra expression in islets and monocytes [75,103], there is a chronic reduction of IL-1Ra and induction of IL-1β secretion.

IL-1Ra expression is increased in white adipose tissue in obese individuals with increased circulating FFA and lipids [104]. In contrast, daily IL-1Ra injections in HFD-fed mice normalizes circulating FFA, lipids as well as adipokines. Although the percentage of macrophages in a given adipose tissue depot is positively correlated with adiposity

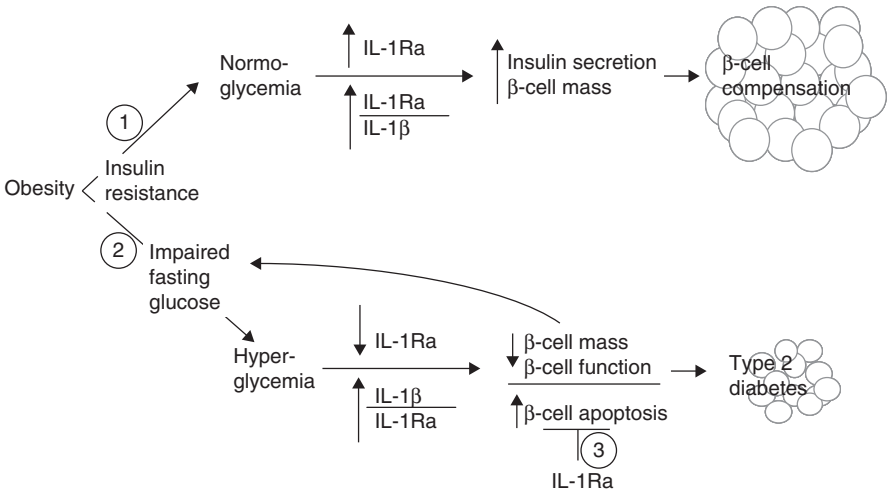

Figure 2. Our hypothetical model illustrating the consequence of obesity for the development of type 2 diabetes. (1) When IL-1Ra is highly expressed in the β-cell and the IL-1Ra/IL-1β balance is towards the protective IL-1Ra, β-cell mass and insulin secretion increase. The β-cell is able to adapt to a situation of higher insulin demand. (2) On the other hand, decreased β-cell expression of IL-1Ra, together with hyperglycemia induced β-cell production of IL-1 β shifts the balance towards the proapoptotic IL-1β, leading to decreased β-cell mass, impaired β -cell function and increased β-cell apoptosis. Glucose levels can no longer be regulated. This results in a vicious cycle and type 2 diabetes develops. (3) Overexpression of IL-1Ra could reverse the process and protect from hyperglycemia induced β-cell apoptosis.

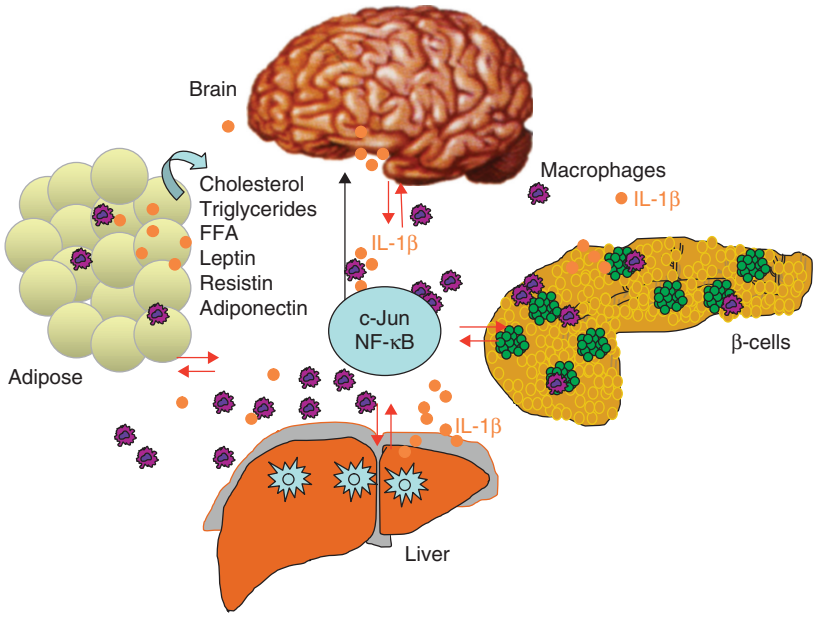

Figure 3. The inflammatory axis in metabolic diseases and interplay between macrophage derived IL-1β and its action in adipose, brain, pancreas and liver tissues. For details see text.
FFA: Free fatty acids.

and adipocyte size [95], the normalization of lipids and adipokines by IL-1Ra seems to be independent of fat mass, since IL-1Ra treatment neither influences fat mass nor adipocyte size. In contrast, mRNA levels of the inflammatory cytokines IL-1β and TNF-α, the macrophage marker F4/80 and the pro-inflammatory macrophage marker CD11c are increased by the HFD in wild-type mice but reduced by IL-1Ra overexpression [87]. Interestingly, specifically the marker of the 'classically activated' macrophages M1 [105] is highly induced by the HFD and normalized by IL-1Ra. Thus, the HFD-induced pro-inflammatory state of adipocytes may be the reason for the increased adipokines (resistin and leptin) and lipid production.

Undoubtedly, the effect of IL-1Ra on adipocyte-derived factors plays a protective role at the level of the β-cell.

7. IL-1β in the liver

The bone marrow–derived macrophage cells in the liver are the Kupffer cells. Kupffer cells secrete cytokines, among them IL-1β, NO and free radicals, which could, by themselves, induce β-cell failure [106]. This is specifically deleterious in the environment of transplanted islets in the liver. Cytokines (IL-1β, IFN-γ and TNF-α) are particularly elevated after islet transplantation [107], and liver tissue macrophages participate in cell injury and graft failure [108,109]. Strategies to inhibit IL-1β-induced β-cell failure, for example by salicylate treatment of the islets [110,111] may therefore improve graft survival.

Similar to the role of macrophages in obese adipose tissue, secretion of IL-1β by the Kupffer cells could be central to hepatic insulin resistance in obesity. Cytokine-induced JNK phosphorylation and activation of the NF-κB pathway are indicative of insulin resistance in the liver, for example depletion of JNK in myeloid cells (including Kupffer cells) in mice leads to HFD-induced hepatic steatosis without an increase in inflammatory markers in the liver and no development of insulin resistance [112]. Furthermore, hepatocyte specific inhibition of NF-κB [113] or of IKKβ [114] in myeloid cells improves hepatic insulin sensitivity. These studies show that independent of obesity, the inflammatory status in the liver primarily regulates insulin sensitivity.

8. IL-1β in the brain

In the healthy brain, members of the IL-1 family are expressed at low or undetectable levels [115]. During neuro-inflammation, IL-1β is dramatically upregulated by various local and systemic brain insults including ischaemia, trauma, hypoxia and neurotoxic inflammatory stimuli [116].

IL-1β in the brain is produced primarily by microglia, which also express caspase 1 [116]. To a lesser extent, astrocytes, oligodendroglia, neurons, cerebrovascular cells and circulating immune cells after infiltrating the brain under inflammatory conditions, produce IL-1β [117].

IL-1β has a number of diverse actions in the CNS to modify feeding behavior, fever [118], central pain modulation [119], stress responses [120] memory [121] and neuroendocrine responses, mainly through actions in the hypothalamus [122].

There is evidence of a hypothalamic control of insulin sensitivity which is disturbed when elevated levels of pro-inflammatory cytokines are circulating. Studies in mice show that HFD promotes hypothalamic resistance to the main anorexigenic hormones, leptin and insulin, leading to the progressive loss of the balance between food intake and thermogenesis and, therefore, resulting in body mass gain [123-125].

Also, the structural and metabolic damage found in Alzheimer`s disease, is in part due to sustained elevation of IL-1β [126-128]. It upregulates expression of β-amyloid precursor protein (β-APP) and stimulates the processing of β-APP resulting in amyloidogenic fragments in neurons [129]. Similarly, the β-APP deposits found in the Alzheimer brain share the same molecular structure as the amylin oligomer deposits found in the pancreatic β-cells in T2DM and are equally neurotoxic [130]. Based on observations in the human islet amyloid polypeptide transgenic rat [131], we have observed IL-1β expression within the islets after the induction of severe hyperglycemia (unpublished observation), indicating that IL-1β expression can only be observed at high glucose levels. It remains to be elucidated if the toxicity of amylin oligomers on the β-cell involves IL-1β signals.

Possibly, the activation of cytokine-induced pro-inflammatory pathways (e.g., JNK) plays a major role in the modulation of neurodegeneration [132]. In line with this hypothesis, JNKs are negatively regulating insulin sensitivity in the obese state.

Four different pathways are shown in the brain as a consequence of diet-induced activation of inflammatory signaling; i) induction of suppressor of cytokine signaling-3 (SOCS3) expression [133]; ii) activation of JNK and IKK [123]; iii) induction of protein tyrosine phosphatase 1B (PTP1B) [134]; and iv) activation of TLR4 signaling [124]. Thus, obesity and HFD induce activation of pro-inflammatory pathways in the brain which may directly develop insulin resistance and lead to diminished glucose regulation by the insulin target tissues.

9. Lessons from the IL-1 mouse models

Having shown the deleterious effects of IL-1β on the β-cell, one would suppose that the IL-1β knockout mouse would be the ideal model for improved β-cell survival and function. Conversely, IL-1β-KO mice show impaired glucose tolerance, decreased β-cell mass and decreased expression of β-cell transcription factors (e.g., PDX-1, Pax-4) [61], indicating that IL-1β has a dual role in the β-cell and activated pathways for example Fas-linked interleukin 1-B converting enzyme-like protease inhibitor protein (FLIP), Fas and NF-κB might be needed for insulin secretion and survival [61,136,137]. In line with these data, Caspase 8-knockout [136] and Fas-deficient mice [137] show impaired glucose tolerance. Especially the dual role of NF-κB, which was long known to be responsible

for IL-1β-induced β-cell destruction [138] is shown through NF-κB-induced activation of the antiapoptotic gene A20, which protects against cell death [139], promotion of insulin secretion [140] and β-cell specific NF-κB depletion accelerates diabetes in the NOD mouse [16]. In contrast and despite their basally impaired glucose tolerance, IL-1β-KO mice are protected against the diabetogenic effects of the HFD as well as against glucotoxicity [61], which supports the concept that IL-β mediates nutrient-induced β-cell dysfunction during the development of T2DM.

In NOD mice, IL-1R deficiency slows but does not prevent diabetes progression [141], and caspase 1 (interleukin converting enzyme) deficiency has no effect on diabetes progression [142], although both IL-1R subtype 1 and caspase 1 are highly expressed in islets from wild type NOD mice [143]. It is possible that pathways other than IL-1β signals are involved in diabetes in NOD mice since it has been shown that IL-10 promotes diabetes in NOD mice independent of Fas, perforin, TNFR 1 and TNFR 2 [144].

10. Blocking IL-1β signals *in vivo* inhibits diabetes progression

Recently, the hypothesis that blocking IL-1β would be a successful strategy for the therapy of T2DM has been proven by several studies. Daily injection of IL-1Ra in mice fed a HFD improved glycemia, glucose stimulated insulin secretion and survival [87], reduced hyperglycaemia and reversed the islet inflammatory phenotype in the GK rat [145]. Treatment with an IL-1β antibody also improved glycemic control in diet-induced obesity in mice [146,147].

Importantly, results from a recent clinical study in patients with T2DM showed that IL-1Ra improved glycemic control and β-cell function [148]. After 13 weeks of treatment, C-peptide secretion was increased and inflammatory markers, for example IL-6 and C-reactive protein were reduced in the IL-1Ra group. HbA1c was significantly lower in the IL-1Ra group compared with the placebo group, which correlated with the body-surface area in the IL-1Ra group. The dose of 100 mg IL-1Ra was given daily to the patients without weight adjustment. Currently ongoing trials, which include dose adjustment to body weight, may result in better glycemic control in the higher body surface area group. The effect of IL-1 antagonism on β-cell function is currently being tested in patients with recent onset of T1DM [149]. Both, IL-1Ra as well as anti-IL-1β antibody Xoma 052 do not completely block IL-1β signaling. While IL-1Ra is a competitive antagonist to IL-1β, XOMA 052 has a novel mechanism of action that reduces IL-1β activity by 40 – 50-fold rather than completely blocking it [146,150]. Given the dual effect of IL-1β on β-cell survival and insulin secretion, this may be an important characteristic of both drugs.

As shown by these recent studies, blocking IL-1β signaling may be a powerful new treatment for T2DM which does not rely on replacing insulin exogenously but acts at the level of the β-cell to improve β-cell survival and to improve endogenous insulin secretion and action. Moreover, blocking IL-1β may also improve insulin sensitivity. Further studies will be necessary to clarify the contradiction of IL-1Ra`s modulation of insulin sensitivity and the impact of IL-β on β-cell survival in T2DM.

11. Expert opinion

To maintain β-cell mass and function to achieve normoglycemia is the major aim for diabetes therapy. Such strategy involves β-cell replacement through islet or pancreas transplantation or triggering their regeneration, a development which is still far away from a general application for diabetes therapy.

In any case, the remaining β-cell mass as well as any newly transplanted or formed β-cells need to be protected from further destruction. Secreted IL-1β into the proximity of the β-cell induces their destruction and impairs insulin secretion. Several recent studies show that intra-islet inflammation contributes to the decreased β-cell mass in T2DM. Blocking such deleterious signals through binding and inactivation of IL-1β may therefore be a successful way towards long-term survival of insulin-producing β-cells.

Declaration of interest

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Bibliography

1. Auron PE, Webb AC, Rosenwasser LJ, et al. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc Natl Acad Sci USA 1984;81:7907-11
2. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009;27:519-50
3. Boraschi D, Tagliabue A. The interleukin-1 receptor family. Vitam Horm 2006;74:229-54
4. Deyerle KL, Sims JE, Dower SK, Bothwell MA. Pattern of IL-1 receptor gene expression suggests role in noninflammatory processes. J Immunol 1992;149:1657-65
5. Scarim AL, Arnush M, Hill JR, et al. Evidence for the presence of type I IL-1 receptors on β-cells of islets of Langerhans. Biochim Biophys Acta 1997;1361(3):313-20
6. Mandrup-Poulsen T, Bendtzen K, Nielsen JH, et al. Cytokines cause functional and structural damage to isolated islets of Langerhans. Allergy 1985;40:424-9
7. Mandrup-Poulsen T, Bendtzen K, Nerup J, et al. Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. Diabetologia 1986;29:63-7
8. Eizirik DL. Interleukin-1 induced impairment in pancreatic islet oxidative metabolism of glucose is potentiated by tumor necrosis factor. Acta Endocrinol (Copenh) 1988;119:321-5
9. Pukel C, Baquerizo H, Rabinovitch A. Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. Diabetes 1988;37:133-6
10. Bendtzen K, Mandrup-Poulsen T, Nerup J, et al. Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. Science 1986;232:1545-7
11. Dinarello CA. Biology of interleukin 1. Faseb J 1988;2:108-15
12. Warner SJ, Auger KR, Libby P. Human interleukin 1 induces interleukin 1 gene expression in human vascular smooth muscle cells. J Exp Med 1987;165:1316-31
13. Frobose H, Ronn SG, Heding PE, et al. Suppressor of cytokine signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. Mol Endocrinol 2006;20:1587-96
14. Mokhtari D, Myers JW, Welsh N. The MAPK kinase kinase-1 is essential for stress-induced pancreatic islet cell death. Endocrinology 2008;149:3046-53
15. Giannoukakis N, Mi Z, Rudert WA, et al. Prevention of beta cell dysfunction and apoptosis activation in human islets by adenoviral gene transfer of the insulin-like growth factor I. Gene Ther 2000;7:2015-22
16. Kim S, Millet I, Kim HS, et al. NF-κB prevents β cell death and autoimmune diabetes in NOD mice. Proc Natl Acad Sci USA 2007;104:1913-8
17. Carpenter L, Cordery D, Biden TJ. Protein kinase Cδ activation by interleukin-1β stabilizes inducible nitric-oxide synthase mRNA in pancreatic β-cells. J Biol Chem 2001;276:5368-74
18. Carpenter L, Cordery D, Biden TJ. Inhibition of protein kinase C δ protects rat INS-1 cells against interleukin-1β and streptozotocin-induced apoptosis. Diabetes 2002;51:317-24
19. Augstein P, Dunger A, Heinke P, et al. Prevention of autoimmune diabetes in NOD mice by troglitazone is associated with modulation of ICAM-1 expression on pancreatic islet cells and IFN-γ expression in splenic T cells. Biochem Biophys Res Commun 2003;304:378-84
20. Stassi G, Todaro M, Richiusa P, et al. Expression of apoptosis-inducing CD95 (Fas/Apo-1) on human beta-cells sorted by flow-cytometry and cultured in vitro. Transplant Proc 1995;27:3271-5
21. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T. Inflammatory mediators and islet β-cell failure: a link between type 1 and type 2 diabetes. J Mol Med 2003;81:455-70
22. Cardozo AK, Ortis F, Storling J, et al. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic β-cells. Diabetes 2005;54:452-61
23. Oyadomari S, Araki E, Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic β-cells. Apoptosis 2002;7:335-45
24. Akerfeldt MC, Howes J, Chan JY, et al. Cytokine-induced β-cell death is independent of endoplasmic reticulum stress signaling. Diabetes 2008;57:3034-44
25. Larsen CM, Wadt KA, Juhl LF, et al. Interleukin-1β-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. J Biol Chem 1998;273:15294-300
26. Welsh N. Interleukin-1β-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. J Biol Chem 1996;271:8307-12
27. Abdelli S, Abderrahmani A, Hering BJ, et al. The c-Jun N-terminal kinase JNK participates in cytokine- and isolation stress-induced rat pancreatic islet apoptosis. Diabetologia 2007;50:1660-9
28. Bonny C, Oberson A, Negri S, et al. Cell-permeable peptide inhibitors of JNK: novel blockers of β-cell death. Diabetes 2001;50:77-82
29. Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL. Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic β-cells. Eur Cytokine Netw 2000;11:267-74
30. Saldeen J, Lee JC, Welsh N. Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. Biochem Pharmacol 2001;61:1561-9
31. Emanuelli B, Glondu M, Filloux C, et al. The potential role of SOCS-3 in the interleukin-1β-induced desensitization of insulin signaling in pancreatic beta-cells. Diabetes 2004;53:S97-103
32. Storling J, Binzer J, Andersson AK, et al. Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. Diabetologia 2005;48:2039-50
33. Aikin R, Maysinger D, Rosenberg L. Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. Endocrinology 2004;145:4522-31
34. Karlsen AE, Ronn SG, Lindberg K, et al. Suppressor of cytokine signaling 3 (SOCS-3) protects β-cells against interleukin-1β- and interferon-γ-mediated toxicity. Proc Natl Acad Sci USA 2001;98:12191-6

35. Ronn SG, Billestrup N, Mandrup-Poulsen T. Diabetes and suppressors of cytokine signaling proteins. Diabetes 2007;56:541-8

36. Ortis F, Cardozo AK, Crispim D, et al. Cytokine-induced proapoptotic gene expression in insulin-producing cells is related to rapid, sustained, and nonoscillatory nuclear factor-κB activation. Mol Endocrinol 2006;20:1867-79

37. Ronn SG, Borjesson A, Bruun C, et al. Suppressor of cytokine signalling-3 expression inhibits cytokine-mediated destruction of primary mouse and rat pancreatic islets and delays allograft rejection. Diabetologia 2008;51:1873-82

38. Narcisse L, Scemes E, Zhao Y, et al. The cytokine IL-1β transiently enhances P2X7 receptor expression and function in human astrocytes. Glia 2005;49:245-58

39. Arnush M, Heitmeier MR, Scarim AL, et al. IL-1 produced and released endogenously within human islets inhibits beta cell function. J Clin Invest 1998;102(3):516-26

40. Lacy PE. The intraislet macrophage and type I diabetes. Mt Sinai J Med 1994;61:170-4

41. Heitmeier MR, Arnush M, Scarim AL, Corbett JA. Pancreatic β-cell damage mediated by β-cell production of IL-1: A novel mechanism for virus-induced diabetes. J Biol Chem 2001;276:11151-8

42. Maedler K, Sergeev P, Ris F, et al. Glucose-induced β-cell production of interleukin-1β contributes to glucotoxicity in human pancreatic islets. J Clin Invest 2002;110:851-60

43. Boni-Schnetzler M, Thorne J, Parnaud G, et al. Increased interleukin (IL)-1β messenger ribonucleic acid expression in beta -cells of individuals with type 2 diabetes and regulation of IL-1β in human islets by glucose and autostimulation. J Clin Endocrinol Metab 2008;93:4065-74

44. Donath MY, Ehses JA, Maedler K, et al. Mechanisms of β-Cell death in type 2 diabetes. Diabetes 2005;54(Suppl 2):S108-13

45. Cnop M, Welsh N, Jonas JC, et al. Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54(Suppl 2):S97-107

46. Elouil H, Cardozo AK, Eizirik DL, et al. High glucose and hydrogen peroxide increase c-Myc and haeme-oxygenase 1 mRNA levels in rat pancreatic islets without activating NFκB. Diabetologia 2005;48:496-505

47. Mine T, Miura K, Okutsu T, et al. Gene expression profile in the pancreatic islets of Goto-Kakizaki (GK) rats with repeated postprandial hyperglycemia. Diabetes 2004;53(Suppl 2):2475A

48. Jorns A, Rath KJ, Bock O, Lenzen S. Beta cell death in hyperglycaemic Psammomys obesus is not cytokine-mediated. Diabetologia 2006;49:2704-12

49. Welsh N, Cnop M, Kharroubi I, et al. Is there a role for locally produced interleukin-1 in the deleterious effects of high glucose or the type 2 diabetes milieu to human pancreatic islets? Diabetes 2005;54:3238-44

50. Maedler K, Spinas GA, Lehmann R, et al. Glucose induces β-cell apoptosis via upregulation of the Fas-receptor in human islets. Diabetes 2001;50:1683-90

51. Corbett JA, Sweetland MA, Wang JL, et al. Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. Proc Natl Acad Sci USA 1993;90:1731-5

52. Giannoukakis N, Rudert WA, Ghivizzani SC, et al. Adenoviral gene transfer of the interleukin-1 receptor antagonist protein to human islets prevents IL-1β-induced β-cell impairment and activation of islet cell apoptosis in vitro. Diabetes 1999;48:1730-6

53. Loweth AC, Watts K, McBain SC, et al. Dissociation between Fas expression and induction of apoptosis in human islets of Langerhans. Diabetes Obes Metab 2000;2:57-60

54. Loweth AC, Williams GT, James RF, et al. Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1β and Fas ligation. Diabetes 1998;47:727-32

55. Mandrup-Poulsen T, Bendtzen K, Nerup J, et al. Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. Diabetologia 1986;29:63-7

56. Mandrup-Poulsen T, Bendtzen K, et al. Cytokines cause functional and structural damage to isolated islets of Langerhans. Allergy 1985;40:424-9

57. Mandrup-Poulsen T, Zumsteg U, Reimers J, et al. Involvement of interleukin 1 and interleukin 1 antagonist in pancreatic β-cell destruction in insulin-dependent diabetes mellitus. Cytokine 1993;5:185-91

58. Rabinovitch A, Sumoski W, Rajotte RV, Warnock GL. Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. J Clin Endocrinol Metab 1990;71:152-6

59. Stassi G, De Maria R, Trucco G, et al. Nitric oxide primes pancreatic β cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. J Exp Med 1997;186:1193-200

60. Schumann DM, Maedler K, Franklin I, et al. The Fas pathway is involved in β-cell secretory function. Proc Natl Acad Sci USA 2007;104:2861-6

61. Maedler K, Schumann DM, Sauter N, et al. Low concentration of interleukin-1β Induces FLICE-inhibitory protein-mediated β-cell proliferation in human pancreatic islets. Diabetes 2006;55:2713-22

62. Spinas GA, Hansen BS, Linde S, et al. Interleukin 1 dose-dependently affects the biosynthesis of (pro)insulin in isolated rat islets of Langerhans. Diabetologia 1987;30:474-80

63. Spinas GA, Mandrup-Poulsen T, Molvig J, et al. Low concentrations of interleukin-1 stimulate and high concentrations inhibit insulin release from isolated rat islets of Langerhans. Acta Endocrinol (Copenh) 1986;113:551-8

64. Spinas GA, Palmer JP, Mandrup-Poulsen T, et al. The bimodal effect of interleukin 1 on rat pancreatic beta-cells – stimulation followed by inhibition – depends upon dose, duration of exposure, and ambient glucose concentration. Acta Endocrinol (Copenh) 1988;119:307-11

65. Dinarello CA. The role of the interleukin-1-receptor antagonist in blocking inflammation mediated by interleukin-1. N Engl J Med 2000;343:732-4

66. Seckinger P, Lowenthal JW, Williamson K, et al. A urine inhibitor of interleukin 1 activity that blocks ligand binding. J Immunol 1987;139:1546-9

67. Seckinger P, Williamson K, Balavoine JF, et al. A urine inhibitor of interleukin 1 activity affects both interleukin 1 alpha and 1 beta but not tumor necrosis factor alpha. J Immunol 1987;139:1541-5

68. Arend WP, Guthridge CJ. Biological role of interleukin 1 receptor antagonist isoforms. Ann Rheum Dis 2000;59(Suppl 1):i60-4

69. Sandberg JO, Andersson A, Eizirik DL, Sandler S. Interleukin-1 receptor antagonist

prevents low dose streptozotocin induced diabetes in mice. Biochem Biophys Res Commun 1994;202:543-8

70. Nicoletti F, Di Marco R, Barcellini W, et al. Protection from experimental autoimmune diabetes in the non-obese diabetic mouse with soluble interleukin-1 receptor. Eur J Immunol 1994;24:1843-7
71. Sandberg JO, Eizirik DL, Sandler S. IL-1 receptor antagonist inhibits recurrence of disease after syngeneic pancreatic islet transplantation to spontaneously diabetic non-obese diabetic (NOD) mice. Clin Exp Immunol 1997;108:314-7
72. Stoffels K, Gysemans C, Waer M, et al. Interleukin-1 receptor antagonist inhibits primary non-function and prolongs graft survival time of xenogeneic islets transplanted in spontaneously diabetic autoimmune NOD mice. Diabetologia 2002;45(Suppl 2):424
73. Tellez N, Montolio M, Estil-Les E, et al. Adenoviral overproduction of interleukin-1 receptor antagonist increases beta cell replication and mass in syngeneically transplanted islets, and improves metabolic outcome. Diabetologia 2007;50:602-11
74. Satoh M, Yasunami Y, Matsuoka N, et al. Successful islet transplantation to two recipients from a single donor by targeting proinflammatory cytokines in mice. Transplantation 2007;83:1085-92
75. Maedler K, Sergeev P, Ehses JA, et al. Leptin modulates β cell expression of IL-1 receptor antagonist and release of IL-1β in human islets. Proc Natl Acad Sci USA 2004;101:8138-43
76. Sandberg JO, Eizirik DL, Sandler S, et al. Treatment with an interleukin-1 receptor antagonist protein prolongs mouse islet allograft survival. Diabetes 1993;42:1845-51
77. Tellez N, Montolio M, Biarnes M, et al. Adenoviral overexpression of interleukin-1 receptor antagonist protein increases beta-cell replication in rat pancreatic islets. Gene Ther 2005;12:120-8
78. Wilson HL, Francis SE, Dower SK, Crossman DC. Secretion of intracellular IL-1 receptor antagonist (type 1) is dependent on P2X7 receptor activation. J Immunol 2004;173:1202-8
79. Glas R, Sauter NS, Schulthess FT, et al. Purinergic P2X7 receptors regulate secretion of interleukin-1 receptor antagonist and beta cell function and survival. Diabetologia 2009. (In Press)
80. Abbatecola AM, Ferrucci L, Grella R, et al. Diverse effect of inflammatory markers on insulin resistance and insulin-resistance syndrome in the elderly. J Am Geriatr Soc 2004;52:399-404
81. Meier CA, Bobbioni E, Gabay C, et al. IL-1 receptor antagonist serum levels are increased in human obesity: a possible link to the resistance to leptin? J Clin Endocrinol Metab 2002;87:1184-8
82. Ruotsalainen E, Salmenniemi U, Vauhkonen I, et al. Changes in inflammatory cytokines are related to impaired glucose tolerance in offspring of type 2 diabetic subjects. Diabetes Care 2006;29:2714-20
83. Salmenniemi U, Ruotsalainen E, Pihlajamaki J, et al. Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome. Circulation 2004;110:3842-8
84. Marculescu R, Endler G, Schillinger M, et al. Interleukin-1 receptor antagonist genotype is associated with coronary atherosclerosis in patients with type 2 diabetes. Diabetes 2002;51:3582-5
85. Herder C, Brunner EJ, Rathmann W, et al. Elevated levels of the anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) precede the onset of type 2 diabetes (Whitehall II Study). Diabetes Care 2008
86. Juge-Aubry CE, Somm E, Giusti V, et al. Adipose tissue is a major source of interleukin-1 receptor antagonist: upregulation in obesity and inflammation. Diabetes 2003;52:1104-10
87. Sauter NS, Schulthess FT, Galasso R, et al. The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. Endocrinology 2008;149:2208-18
88. Koenig W, Khuseyinova N, Baumert J, et al. Increased concentrations of C-reactive protein and IL-6 but not IL-18 are independently associated with incident coronary events in middle-aged men and women: results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. Arterioscler Thromb Vasc Biol 2006;26:2745-51
89. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111-19
90. de Luca C, Olefsky JM. Inflammation and insulin resistance. FEBS Lett 2008;582:97-105
91. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 2008;118:2992-3002
92. Ehses JA, Perren A, Eppler E, et al. Increased number of islet-associated macrophages in type 2 diabetes. Diabetes 2007;56:2356-70
93. Homo-Delarche F, Calderari S, Irminger JC, et al. Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat. Diabetes 2006;55:1625-33
94. Weksler-Zangen S, Raz I, Lenzen S, et al. Impaired glucose-stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat. Diabetes 2008;57:279-87
95. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 2006;7:85-96
96. Rui L, Yuan M, Frantz D, et al. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 2002;277:42394-8
97. Weisberg SP, McCann D, Desai M, et al. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796-808
98. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003;112:1821-30
99. Koerner A, Kratzsch J, Kiess W. Adipocytokines: leptin–the classical, resistin–the controversical, adiponectin–the promising, and more to come. Best Pract Res Clin Endocrinol Metab 2005;19:525-46
100. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 2006;6:772-83
101. Rasouli N, Kern PA. Adipocytokines and the metabolic complications of obesity. J Clin Endocrinol Metab 2008;93:S64-73
102. Yu X, Park BH, Wang MY, et al. Making insulin-deficient type 1 diabetic rodents thrive without insulin. Proc Natl Acad Sci USA 2008;105:14070-5
103. Seufert J, Kieffer TJ, Leech CA, et al. Leptin suppression of insulin secretion

and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. J Clin Endocrinol Metab 1999;84:670-6

104. Roduit R, Thorens B. Inhibition of glucose-induced insulin secretion by long-term preexposure of pancreatic islets to leptin. FEBS Lett 1997;415:179-82
105. Gabay C, Dreyer M, Pellegrinelli N, et al. Leptin directly induces the secretion of interleukin 1 receptor antagonist in human monocytes. J Clin Endocrinol Metab 2001;86:783-91
106. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007;117:175-84
107. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. J Leukoc Biol 2005;77:587-97
108. Bottino R, Fernandez LA, Ricordi C, et al. Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. Diabetes 1998;47:316-23
109. Kaufman DB, Platt JL, Rabe FL, et al. Differential roles of Mac-1+ cells, and CD4+ and CD8+ T lymphocytes in primary nonfunction and classic rejection of islet allografts. J Exp Med 1990;172:291-302
110. Kaufman DB, Gores PF, Field MJ, et al. Effect of 15-deoxyspergualin on immediate function and long-term survival of transplanted islets in murine recipients of a marginal islet mass. Diabetes 1994;43:778-83
111. Tran PO, Gleason CE, Robertson RP. Inhibition of interleukin-1beta-induced COX-2 and EP3 gene expression by sodium salicylate enhances pancreatic islet beta-cell function. Diabetes 2002;51:1772-8
112. Zeender E, Maedler K, Bosco D, et al. Pioglitazone and Sodium Salicylate Protect Human {beta}-Cells against Apoptosis and Impaired Function Induced by Glucose and Interleukin-1{beta}. J Clin Endocrinol Metab 2004;89:5059-66
113. Solinas G, Vilcu C, Neels JG, et al. JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. Cell Metab 2007;6:386-97
114. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 2005;11:183-90
115. Arkan MC, Hevener AL, Greten FR, et al. IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med 2005;11:191-8
116. Allan SM, Tyrrell PJ, Rothwell NJ. Interleukin-1 and neuronal injury. Nat Rev Immunol 2005;5:629-40
117. Touzani O, Boutin H, Chuquet J, Rothwell N. Potential mechanisms of interleukin-1 involvement in cerebral ischaemia. J Neuroimmunol 1999;100:203-15
118. Rothwell NJ, Luheshi GN. Interleukin 1 in the brain: biology, pathology and therapeutic target. Trends Neurosci 2000;23:618-25
119. Dinarello CA, Wolff SM. Molecular basis of fever in humans. Am J Med 1982;72:799-819
120. Wolf G, Yirmiya R, Goshen I, et al. Impairment of interleukin-1 (IL-1) signaling reduces basal pain sensitivity in mice: genetic, pharmacological and developmental aspects. Pain 2003;104:471-80
121. Goshen I, Yirmiya R, Iverfeldt K, Weidenfeld J. The role of endogenous interleukin-1 in stress-induced adrenal activation and adrenalectomy-induced adrenocorticotropic hormone hypersecretion. Endocrinology 2003;144:4453-8
122. Schneider H, Pitossi F, Balschun D, et al. A neuromodulatory role of interleukin-1beta in the hippocampus. Proc Natl Acad Sci USA 1998;95:7778-83
123. Sims JE, Dower SK. Interleukin-1 receptors. Eur Cytokine Netw 1994;5:539-46
124. De Souza CT, Araujo EP, Bordin S, et al. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology 2005;146:4192-9
125. Milanski M, Degasperi G, Coope A, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci 2009;29:359-70
126. Munzberg H, Flier JS, Bjorbaek C. Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 2004;145:4880-9
127. Vandenabeele P, Fiers W. Is amyloidogenesis during Alzheimer's disease due to an IL-1-/IL-6-mediated 'acute phase response' in the brain? Immunol Today 1991;12:217-19
128. Holden RJ, Mooney PA. Interleukin-1 beta: a common cause of Alzheimer's disease and diabetes mellitus. Med Hypotheses 1995;45:559-71
129. Zuliani G, Ranzini M, Guerra G, et al. Plasma cytokines profile in older subjects with late onset alzheimer's disease or vascular dementia. J Psychiatr Res 2007;41:686-93
130. Goldgaber D, Harris HW, Hla T, et al. Interleukin 1 regulates synthesis of amyloid beta-protein precursor mRNA in human endothelial cells. Proc Natl Acad Sci USA 1989;86:7606-10
131. Haataja L, Gurlo T, Huang CJ, Butler PC. Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis. Endocr Rev 2008;29:303-16
132. Butler AE, Jang J, Gurlo T, et al. Diabetes due to a progressive defect in beta-cell mass in rats transgenic for human islet amyloid polypeptide (HIP Rat): a new model for type 2 diabetes. Diabetes 2004;53:1509-16
133. Borsello T, Forloni G. JNK signalling: a possible target to prevent neurodegeneration. Curr Pharm Des 2007;13:1875-86
134. Howard JK, Cave BJ, Oksanen LJ, et al. Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med 2004;10:734-8
135. Bence KK, Delibegovic M, Xue B, et al. Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med 2006;12:917-24
136. Liadis N, Salmena L, Kwan E, et al. Distinct in vivo roles of caspase-8 in beta-cells in physiological and diabetes models. Diabetes 2007;56:2302-11
137. Schumann DM, Maedler K, Franklin I, et al. The Fas pathway is involved in pancreatic {beta} cell secretory function. Proc Natl Acad Sci USA 2007;104:2861-6
138. Flodstrom M, Welsh N, Eizirik DL. Cytokines activate the nuclear factor kappa B (NF-kappa B) and induce nitric oxide

production in human pancreatic islets. FEBS Lett 1996;385:4-6

139. Liuwantara D, Elliot M, Smith MW, et al. Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. Diabetes 2006;55:2491-501

140. Hammar E, Parnaud G, Bosco D, et al. Extracellular matrix protects pancreatic {beta}-cells against apoptosis: role of short- and long-term signaling pathways. Diabetes 2004;53:2034-41

141. Thomas HE, Irawaty W, Darwiche R, et al. IL-1 receptor deficiency slows progression to diabetes in the NOD mouse. Diabetes 2004;53:113-21

142. Schott WH, Haskell BD, Tse HM, et al. Caspase-1 is not required for type 1 diabetes in the NOD mouse. Diabetes 2004;53:99-104

143. Jafarian-Tehrani M, Amrani A, Homo-Delarche F, et al. Localization and characterization of interleukin-1 receptors in the islets of Langerhans from control and nonobese diabetic mice. Endocrinology 1995;136:609-13

144. Balasa B, La Cava A, Van Gunst K, et al. A mechanism for IL-10-mediated diabetes in the nonobese diabetic (NOD) mouse: ICAM-1 deficiency blocks accelerated diabetes. J Immunol 2000;165:7330-7

145. Ehses JA, Giroix M-H, Coulaud J, et al. IL-1β-MyD88 signaling is central to islet chemokine secretion in response to metabolic stress: evidence from a spontaneous model of type 2 diabetes, the GK rat. Diabetologia 2008;50(Suppl 1):S177

146. Owyang AM, Gross L, Yin J, et al. XOMA 052, an anti-IL-1β antibody, improves glucose control, β-Cell function, and insulin resistance in the diet-induced obesity mouse model. Diabetes 2009;58(S1):A82

147. Osborn O, Brownell SE, Sanchez-Alavez M, et al. Treatment with an Interleukin 1 beta antibody improves glycemic control in diet-induced obesity. Cytokine 2008;44:141-8

148. Larsen CM, Faulenbach M, Vaag A, et al. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med 2007;356:1517-26

149. Pickersgill LM, Mandrup-Poulsen TR. The anti-interleukin-1 in type 1 diabetes action trial--background and rationale. Diabetes Metab Res Rev 2009;25:321-4

150. Donath MY, Weder C, Brunner A, et al. XOMA 052, a potential disease modifying anti-IL-10 antibody shows sustained HbA1c reductions 3 months after single injection with no increases in safety parameters in subjects with T2DM. Diabetes 2008;58S1:A30

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Neutralizing Interleukin-1β (IL-1β) Induces β-Cell Survival by Maintaining PDX1 Protein Nuclear Localization*[S]

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The transcription factor PDX1 plays a critical role during β-cell development and in glucose-induced insulin gene transcription in adult β-cells. Acute glucose exposure leads to translocalization of PDX1 to the nucleoplasm, whereas under conditions of oxidative stress, PDX1 shuttles from the nucleus to the cytosol. Here we show that cytosolic PDX1 expression correlated with β-cell failure in diabetes. In isolated islets from patients with type 2 diabetes and from diabetic mice, we found opposite regulation of insulin and PDX1 mRNA; insulin was decreased in diabetes, but PDX1 was increased. This suggests that elevated PDX1 mRNA levels may be insufficient to regulate insulin. In diabetic islets, PDX1 protein was localized in the cytosol, whereas in non-diabetic controls, PDX1 was in the nucleus. In contrast, overexpression of either IL-1 receptor antagonist or shuttling-deficient PDX1 restored β-cell survival and function and PDX1 nuclear localization. Our results show that nuclear localization of PDX1 is essential for a functional β-cell and provides a novel mechanism of the protective effect of IL-1 receptor antagonist on β-cell survival and function.

New therapies for diabetes that lead to protection of the insulin-producing β-cell are urgently needed. Only when the β-cell compensates for the higher insulin demand during insulin resistance can normoglycemia be maintained. Recent studies suggest that the low grade inflammation in type 2 diabetes mellitus (T2DM)[3] contributes to β-cell failure (1). Especially, interleukin-1β (IL-1β), whose secretion has been postulated from intraislet macrophages (2) and from β-cells themselves when exposed to double-stranded RNA (3), to elevated glucose concentrations (4–6), or to free fatty acids (7), initiates β-cell destruction.

The receptor for IL-1β, IL-1R1, is highly expressed in the β-cell, and more than 10-fold higher expression of IL-1R1 mRNA was observed in isolated islets than in total pancreas, which is attributed to the expression in β-cells (7). This may explain the high sensitivity of the β-cell to IL-1β. A recent study shows that glucose-induced IL-1β secretion involves caspase 1 activation mediated by the NALP3 inflammasome. The inflammasome is activated by bacterial toxins or endogenous stress signals (*e.g.* ATP and β-amyloid (8–10)) through the formation of reactive oxygen species (6, 11). Glucose-induced IL-1β secretion by the β-cell is prevented in NALP3$^{-/-}$ mice, indicating that IL-1β is generated through glucose-induced reactive oxygen species production and oxidative stress (6). The thioredoxin-interacting protein, which has been linked to insulin resistance (12), functions as an activator of NALP3. In line with these data, another recent study shows that thioredoxin-interacting protein is highly increased by elevated glucose in β-cells and that thioredoxin-interacting protein-deficient islets are protected against glucose toxicity (13). Although glucose-induced IL-1β production in the β-cell was not observed in all studies (14), IL-1β expression in islets from patients with T2DM has been confirmed (5, 15), supporting the concept of blocking IL-1β signals as a target for diabetes treatment. Indeed, daily injection of IL-1Ra in mice fed a high fat diet (HFD) improves glycemia, glucose-stimulated insulin secretion, and survival (16); reduces hyperglycemia; and reverses the islet inflammatory phenotype in the GK rat (17). Treatment with an IL-1β antibody also improves glycemic control in diet-induced obesity in mice (18, 19). Results from a clinical study in patients with T2DM showed that IL-1Ra improves glycemic control and β-cell function (20–22). Blocking IL-1β signals reduces expression of inflammatory marker in fat tissue and in islets (16). None of the studies have studied the mechanisms of the protective effect of IL-1Ra at the level of β-cell gene regulation. IL-1β affects expression of the transcription factor PDX1 (pancreatic duodenal homeobox-1, previously called IPF1, IDX1, STF1, or IUF1) (23), a key factor in pancreas development and function. Reduced PDX1 expression levels negatively regulates insulin expression and secretion and predispose islets to apoptosis (24–26). Its homozygous mutations result in

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[3] The abbreviations used are: T2DM, type 2 diabetes mellitus; HFD, high fat diet; ND, normal diet; NES, nuclear export signal; IL-1Ra, IL-1 receptor antagonist; PARP, poly(ADP-ribose) polymerase; OE, overexpressing; JNKi, JNK inhibitor.

pancreas agenesis associated with neonatal diabetes (27, 28). PDX1 directly binds to the promoter and controls expression of important β-cell genes, which are vital for β-cell function, such as insulin, Glut2, and glucokinase (27). PDX1 deficiency contributes to impaired proliferation and enhanced apoptosis via transcriptional mechanisms in models of type 2 diabetes, such as *Psammomys obesus* and the leptin receptor-deficient ($Lepr^{db/db}$) db/db mice (29). Overexpression of PDX1 restores β-cell mass and function, thereby preventing the onset of diabetes in IRS2 knock-out mice, showing the critical role of PDX1 in β-cell survival (30).

Complicated signaling networks control PDX1 regulation, and nucleo-cytoplasmic shuttling plays a major role in the regulation of PDX1 function (31, 32). Well characterized PDX1 nuclear import signal and nuclear export signal (NES) suggest that PDX1 might be regulated at the level of cellular localization (31, 33). Post-translational modification of proteins is the most abundant form of cellular regulation, affecting many cellular signal pathways, including metabolism, growth, differentiation, and apoptosis. In response to acute elevation of glucose and survival factors, such as insulin, PDX1 is phosphorylated and translocates to the nucleus (34, 35). By contrast, stimuli associated with diabetes, such as oxidative stress (31) and free fatty acids (36), cause nuclear exclusion of PDX1 (36). This suggests that cytoplasmic accumulation may represent a mechanism to reduce the nuclear action of PDX1 under pathologic conditions rather than to promote a specific cytoplasmic function.

We have shown previously that a diet enriched with fat and sucrose ("Surwit"; HFD) induces impaired glucose tolerance after 4 weeks of feeding, impaired fasting glucose after 8 weeks, and hyperglycemia after 12 weeks in C57Bl/6J mice (16, 19, 37). These changes in glycemia were accompanied by fluctuations in β-cell mass. Despite the reduction in β-cell proliferation and the increase in β-cell apoptosis, islets showed a compensatory increase in β-cell mass up to 12 weeks of diet. After 16 weeks, apoptosis was increased, and β-cell mass was reduced in the HFD-treated mice. IL-1β antagonism by anti-IL-1β antibody treatment, IL-1Ra injections, or overexpression restored normoglycemia and β-cell function and survival (16, 19, 37), but also changes in the inflammatory state of the fat tissue were involved in the protective effects of IL-1β antagonism. Therefore, we asked how IL-1β signals regulate gene transcription in the β-cell. We tested the effect of IL-1Ra on regulating glucose homeostasis in another animal model, the obese diabetic leptin receptor-deficient $Lepr^{db/db}$ mice (db/db). Commonly, we detected that IL-1Ra was able to maintain the cellular localization of PDX1. A diabetic milieu *in vitro* as well as T2DM *in vivo* induced a switch of PDX1 from the nucleus to the cytosol, which was accompanied by a loss in β-cell mass and function.

Whether PDX1 is altered in its localization in β-cells during the progression to diabetes and whether these changes may affect β-cell function in different levels was previously unknown. Because PDX1 regulates insulin and specific β-cell genes, altered localization may contribute to β-cell death and loss of function. In the short term, this stressful response may be tolerated, but under chronic situations in T2DM, prolonged stress conditions ultimately affect β-cell survival.

EXPERIMENTAL PROCEDURES

Animals—Transgenic mice overexpressing IL-1Ra (IL-1Ra-OE) were kindly provided by Dr. Emmet Hirsch (Northwestern University, Evanston, IL) (38). Beginning at 5 weeks of age and continuing for 12 weeks, transgenic animals as well as their wild type littermates were fed a normal diet (WT ND and IL-1Ra-OE ND) or a high fat/high sucrose diet (WT HFD and IL-1Ra-OE HFD, Surwit (Research Diets, New Brunswick, NJ), containing 58, 26, and 16% calories from fat, carbohydrate, and protein, respectively (39)) or were treated daily with IL-1Ra from the starting day of the diets (ND+IL-1Ra and HFD+IL-1Ra; Kineret®, Amgen (Thousand Oaks, CA), intraperitoneal injection of 10 mg/kg body weight) as described previously (16). Four independent experiments with a total of 16 mice (4 mice/cage) in each group were performed, respectively.

Heterozygous leptin receptor deficient mice on the C57BLKS/J background ($Lepr^{db/+}$, $db/^{+}$) were purchased from Jackson Laboratory. By cross-breeding these mice to C57BL/6J-IL-1Ra-overexpressing mice (OE), we obtained diabetic $Lepr^{db/db}$ (db/db) as well as non-diabetic $Lepr^{+/+}$ (WT) with endogenous overexpression of IL-1Ra (OE-db/db and OE) as well as littermates without IL-1Ra as appropriate negative controls (db/db and WT) with the same mixed background. In all experiments, littermates from the F2 breeding were used. All animals were housed in a temperature-controlled room with a 12-h light/dark cycle and were allowed free access to food and water in agreement with National Institutes of Health animal care guidelines and Section 8 of the German animal protection law.

Intraperitoneal Glucose and Insulin Tolerance Tests—After 4, 8, and 12 weeks of diet (HFD) or at the age of 6 and 10 weeks (db/db), all animals underwent *in vivo* studies (intraperitoneal glucose and insulin tolerance tests) as described before (16).

Islet Isolation and Culture—Islets from all groups were isolated as described previously (40). Human islets were isolated from eight pancreases of healthy organ donors and from eight with T2DM at the University of Illinois (Chicago, IL), Lille University, or Pisa University as described previously (41) and cultured in CMRL-1066 medium as described previously (42). Islet purity was greater than 95% as judged by dithizone staining (if this degree of purity was not achieved by routine isolation, islets were hand-picked). Islets were exposed to 5.5, 11.1, 22.2, or 33.3 mM glucose or 5.5 mM plus 2 ng/ml recombinant human IL-1β (R&D Systems, Minneapolis, MN), with or without 500 ng/ml recombinant human IL-1Ra (R&D Systems) or 10 μM JNKi (kindly provided by Xigen S.A., Lausanne, Switzerland) for 72 h.

Transfections—At 2 days postisolation and culture on extracellular matrix-coated dishes, isolated islets were exposed to transfection Ca^{2+}-KRH medium (4.74 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgCl_26H_2O$, 119 mM NaCl, 2.54 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES). After 1 h of incubation, lipoplexes (Lipofectamine2000 (Invitrogen)/DNA ratio 2.5:1, 5 μg of DNA/100 islets) were added to transfect the islets. After an additional 6 h of incubation, CMRL 1066 medium containing 20% FCS and L-glutamine was added to the transfected islets. Efficient transfection was evaluated based on enhanced GFP-positive cells, which resulted in 60% transfection efficiency in

β-cells through the whole islets, analyzed by fluorescence and confocal microscopy.

Glucose-stimulated Insulin Secretion and Insulin Content—Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel) for 4 days, allowing the cells to attach to the dishes and spread (43). These experimental conditions allowed direct comparison with our previous studies in human and mouse islets pretreated with IL-1Ra *in vitro* (4, 16). Thereafter, islets were washed with PBS and extracted with HCl (0.18 N) in 70% ethanol for 24 h at 4 °C. The acid-ethanol extracts were collected for determination of insulin content. Insulin was determined using a human insulin ELISA kit (Alpco).

β-Cell Mass and Histochemical Analyses—Pancreatic sections from seven healthy controls and from seven patients with T2DM were obtained from the National Disease Research Interchange (NDRI) and from Kangnam St. Mary's Hospital (Seoul, Korea) as described (44); approval for the studies was granted by the Kangnam St. Mary's Hospital and by the Ethical Commission of Bremen University.

Mouse pancreases and sections from isolated islets were obtained as described (16). To determine β-cell mass, 10 sections (spanning the width of the pancreas) were analyzed as described (16). For detection of β-cell apoptosis, insulin and TUNEL staining were performed (In Situ Cell Death Detection Kit, TMR Red; Roche Applied Science) (16). For PDX1 localization studies, after dehydration, sections were incubated in blocking buffer containing 0.2% Tween 20, 3% IgG-free bovine serum albumin (BSA), 2% Triton X-100 for 1 h at room temperature and overnight at 4 °C with rabbit anti-PDX1 (kindly provided by Christopher Wright, Vanderbilt University Medical Center, Nashville, TN) in antibody buffer containing 0.2% Tween 20, 3% IgG-free BSA, and 0.5% Triton X-100. Subsequently, all sections were double-stained for insulin and detected by donkey anti-guinea pig FITC-conjugated antibody (Dako). Fluorescent slides were analyzed using a Nikon MEA53200 microscope (Nikon GmbH Dusseldorf, Germany), and images were acquired using NIS-Elements software (Nikon).

RNA Extraction and RT-PCR Analysis—Total RNA of isolated islets was extracted after overnight culture, and RT-PCR was performed as described previously (4). Primers used were as follows: 5′-ttcttctacacaccca-3′/5′-ctagttgcagtagttct-3′ (human and mouse insulin); 5′-gaggacccgtactgcctaca-3′/5′-cggggtcccgctactacgtt-3′ (mouse PDX1), 5′-ctggattggcgttgtttgtg-3′/5′-tcccaaggtggagtgctgtag-3′ (human PDX1), 5′-gtccatgccatcactgccac-3′/5′-cagcaccagtggatgcaggg-3′ (mouse GAPDH); 5′-gttggccaggctggtgtccag-3/5-ctgtgatgagctgctcagggtgg-3 (human and mouse tubulin), 5′-ccaaccgcgagaagatga-3′/5′-ccagaggcgtacagggatag-3′ (human actin), and 5′-tacgggtcctggcatcttgt-3′/5′-ccatttgtgttgggtccagc-3′ (human cyclophilin).

Nuclear Fractionation—Nuclear and cytoplasmic extractions of human islets were performed according to the instructions for the NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The purity of fractions was analyzed by incubation of the membranes with anti-tubulin and anti-GAPDH for cytosolic and anti-PARP or anti-histone H3 for nuclear extracts.

Western Blot Analysis—At the end of the incubation periods, islets were washed in ice-cold PBS and lysed as described (42). Membranes were incubated with rabbit anti-phospho-JNK (Thr^{183}/Tyr^{185}), rabbit anti-histone H3, rabbit anti-PARP, rabbit anti-tubulin, rabbit anti-GAPDH, and rabbit anti-β-actin (Cell Signaling Technology) antibodies, followed by horseradish peroxidase-linked anti-rabbit IgG. Density of the bands was analyzed using DocIT®LS image acquisition 6.6a (UVP BioImaging Systems, Upland, CA).

Statistical Analysis—Samples were evaluated in a randomized manner by a single investigator, who was blinded to the treatment conditions. Data are presented as means ± S.E. and were analyzed by paired, Student's *t* test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

RESULTS

High Fat Diet Induces PDX1 Translocalization to the Cytosol and Is Prevented by IL-1Ra—Looking into detailed mechanisms of the loss of adaption in C57Bl/6J mice fed a diet enriched with fat and sucrose (HFD; Surwit), we isolated RNA from islets from mice after 12 weeks of diet and IL-1Ra treatment and performed RT-PCR for insulin and its transcription factor PDX1. Whereas insulin mRNA in islets from HFD animals was decreased to 27% of control islets (Fig. 1*A*), PDX1 levels were 2.8-fold increased (Fig. 1*C*). Also, insulin content from isolated islets was significantly decreased in the HFD-fed mice (Fig. 1*B*, $p < 0.05$). Such changes did not occur in IL-1Ra-treated animals. Because it has been reported that PDX1 activity is primarily regulated by its subcellular localization (45, 46), we stained pancreatic tissue sections for PDX1 and insulin. Sections from both normal diet groups showed PDX1 immunoreactivity predominantly (95 and 94%) in the nucleus of β-cells (Fig. 1*D*, *b* and *d*). In contrast, the majority (67%) of the β-cells under the high fat diet expressed PDX1 in the cytoplasm (Fig. 1*C*, *f*). IL-1Ra treatment inhibited this translocalization to the cytoplasm (30% cytosolic PDX1), and the islets showed more prominent staining in the nucleus (54% nuclear PDX1; Fig. 1*D*, *h*).

Western blot analysis from nuclear and cytosolic fractions from isolated islets from WT and IL-1Ra-OE mice fed for 12 weeks with normal chow or HFD confirmed the decrease in nuclear together with the increase in cytosolic PDX1 in islets under the HFD condition, which was prevented by IL-1Ra overexpression (Fig. 1*E*).

PDX1 Translocalization to the Cytosol in db/db Mice Is Prevented by IL-1Ra Overexpression—To investigate whether PDX1 shuttling also occurs in another mouse model, we examined the effects of IL-1Ra in $Lepr^{db/db}$ mice (db/db). By cross-breeding mice, which endogenously overexpress IL-1Ra (OE) to C57BLKS-$Lepr^{db}$, we used four groups of mice in the experiments: IL-1Ra-OE-$Lepr^{db/db}$ (db/dbIL-1Ra) and heterozygous $Lepr^{db/+}$-IL-1Ra-OE (db/$^{+}$IL-1Ra) as well as their littermates without IL-1Ra-overexpression, $Lepr^{db/db}$ (db/db) and $Lepr^{db/+}$ (db/$^{+}$).

Heterozygous db/$^{+}$ and db/$^{+}$IL-1Ra mice showed clear nuclear PDX1 localization independent of their age (84 and 87%

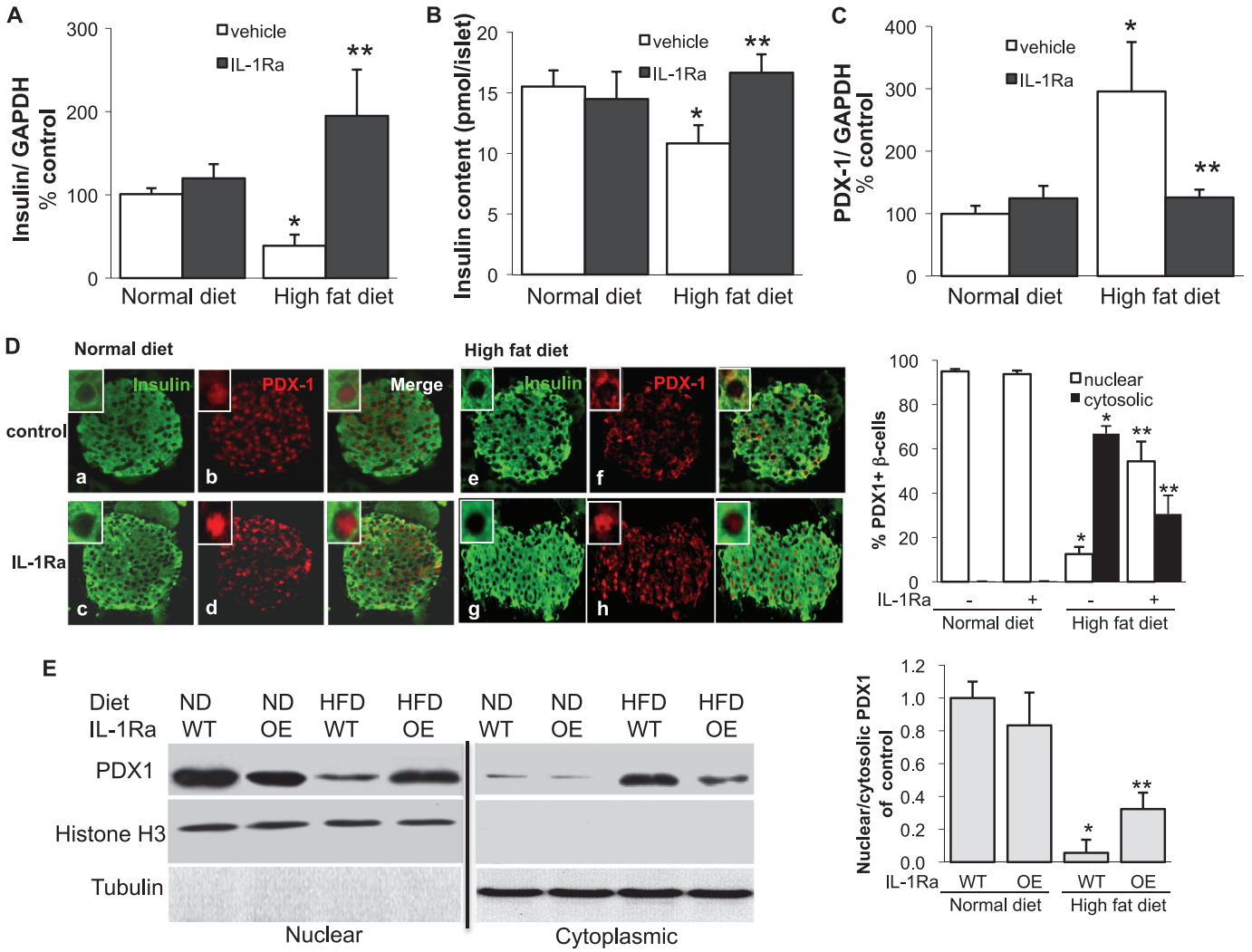

FIGURE 1. **High fat diet induces PDX1 translocalization to the cytosol.** C57Bl/6J mice treated daily with vehicle or 10 mg/kg IL-1Ra were fed a diet enriched with fat and sucrose (HFD) or a normal chow diet (ND) for 12 weeks. *A–C*, RT-PCR analysis of insulin (*A*) and PDX1 (*C*) expression relative to control normal diet conditions and insulin content (*B*) of mouse islets isolated from the four treatment groups. In the LightCycler System, mRNA levels were normalized to GAPDH and tubulin with the same result. Islets were isolated from four mice per treatment group. *D*, double immunostaining for insulin (*green*; *a*, *c*, *e*, and *g*) and PDX1 (*red*; *b*, *d*, *f*, and *h*) in mouse pancreatic tissue sections from all four treatment groups; staining was performed on four different pancreases per treatment group (magnification, ×250; *inset* magnification, ×2000). The percentage of nuclear and cytosolic PDX1 was calculated by counting 2000 insulin-positive β-cells from four mice in each condition. *E*, Western blot analysis of PDX1 from nuclear and cytosolic fractions from isolated islets from C57Bl/6J WT- or IL-1Ra-overexpressing mice fed an ND or HFD for 12 weeks. Histone H3 and tubulin were used as loading controls, and purity of fractions for the nuclear and cytosolic extracts from the same protein lysates was assessed. One representative blot of three experiments is shown. Densitometry analysis of bands normalized to histone H3 or tubulin shows the ratio of nuclear and cytosolic PDX1 expression. Data are shown as mean ± S.E. *, $p < 0.05$ HFD compared to ND WT mice, **, $p < 0.05$ IL-1Ra treated HFD compared to nontreated HFD mice.

nuclear PDX1; Fig. 2*A*, *a–f*). In contrast, 6-week-old db/db mice showed a clear switch of PDX1 from the nucleus into the cytosol (Fig. 2*A*, *g–i*). Littermates, which overexpressed IL-1Ra, were clearly protected from such localization change (Fig. 2*A*, *j–l*). The switch in localization was predominant in the 6-week-old mice. With the progression of diabetes in 10-week-old mice, loss of PDX1 expression was observed (only 15% PDX1 + β-cells; Fig. 2*A*, *m–o*). IL-1Ra-OE mice partly restored overall PDX1 expression and its nuclear localization (51% β-cells had nuclear and 12% cytosolic PDX1; Fig. 2*A*, *p–r*). The localization switch was again confirmed by Western blot analysis in 6-week-old mice. Although heterozygous db/$^+$ and db/$^+$IL-1Ra as well as db/dbIL-1Ra mice showed major nuclear PDX1 localization, we found PDX1 predominantly in the cytosol in the db/db mice (Fig. 2*B*).

The switch in the PDX1 localization to the cytosol was accompanied by impaired glucose tolerance. Although db/$^+$IL-1Ra and db/$^+$ mice showed normal glucose tolerance, db/db mice revealed increased fasting glucose levels and impaired glucose tolerance. IL-1Ra overexpression in the db/$^+$IL-1Ra mice could partially but significantly restore glucose tolerance at all time points during the intraperitoneal glucose tolerance test and decrease fasting glucose levels (supplemental Fig. 1*A*). Glucose-stimulated insulin secretion was completely abolished in the db/db mice but was significantly restored in the db/dbIL-1Ra mice with the significant increase in stimulated insulin secretion and a restoration of the stimulatory index (supplemental Fig. 1, *B* and *C*). β-Cell failure seen by the impaired GSIS was also confirmed when we analyzed β-cell mass and survival. β-Cell apoptosis was 4.5-fold

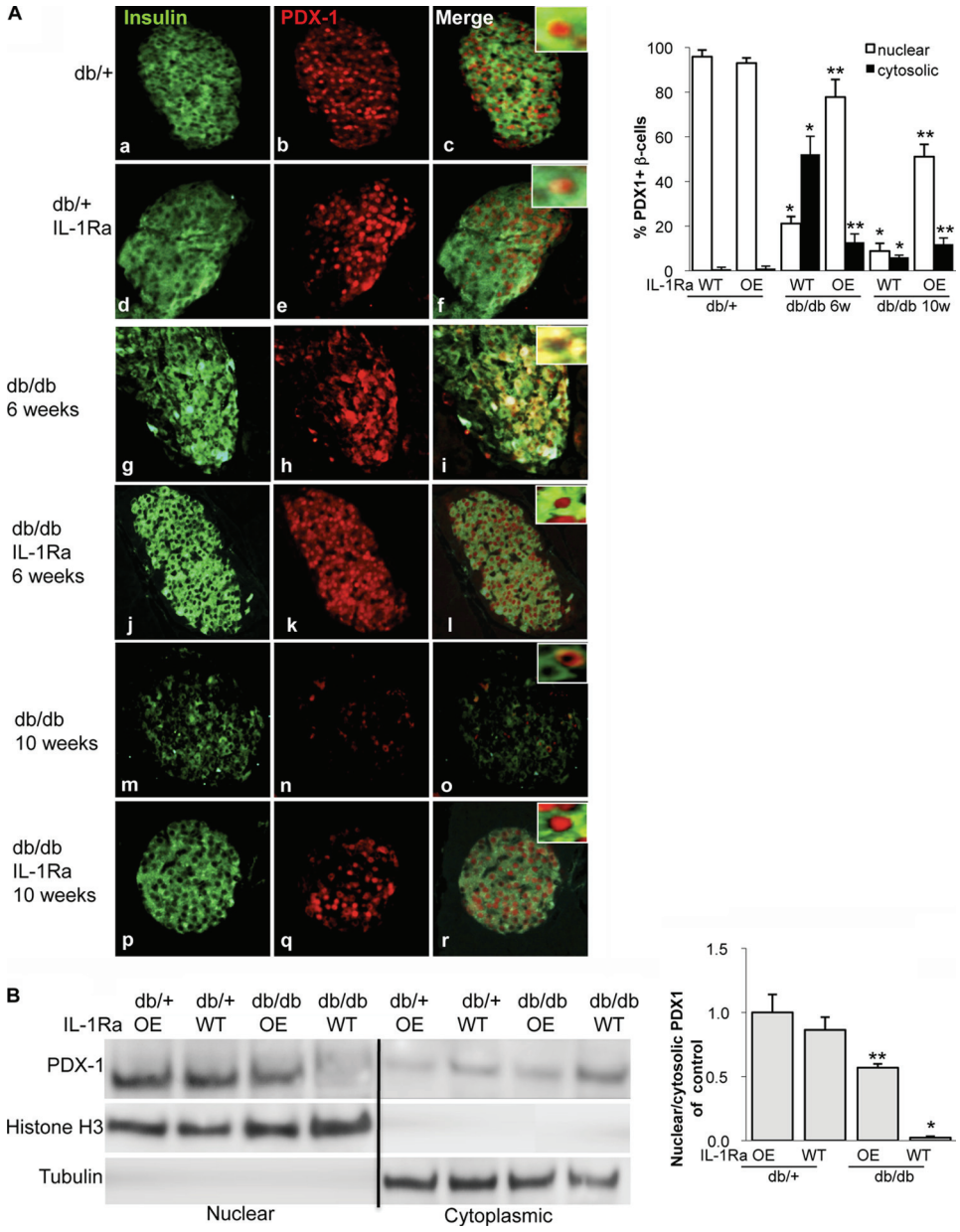

FIGURE 2. **PDX1 translocalization to the cytosol in db/db mice is prevented by IL-1Ra overexpression.** *A*, insulin staining in *green* (*a*, *d*, *g*, *j*, *m*, and *p*) and PDX1 in *red* (*b*, *e*, *h*, *k*, *n*, and *q*) of control heterozygous db/$^{+}$ (*a–c*) and db/$^{+}$IL-1Ra (*d–f*) mice and db/db (*g–i* and *m–o*) and db/dbIL-1Ra (*j–l* and *p–r*) littermates at the age of 6 (*g–l*) and 10 weeks (*m–r*). Staining was performed on three different pancreases per treatment group from three independent experiments, respectively (magnification, ×250; *inset* magnification, ×2000). Percentage of nuclear and cytosolic PDX1 was calculated by counting 2000 insulin-positive β-cells from three mice in each condition. *B*, Western blot analysis of islet lysates from 6-week-old heterozygous db/$^{+}$, db/$^{+}$IL-1Ra, db/db, and db/dbIL-1Ra mice. Histone H3 and tubulin were used as loading controls, and purity of fractions in the same membrane after stripping was assessed. One representative blot of three experiments is shown. Densitometry analysis of bands normalized to histone H3 or tubulin shows the ratio of nuclear and cytosolic PDX1 expression. Data are shown as mean ± S.E. *, $p < 0.05$ db/db compared to db/$^{+}$, **, $p < 0.05$ OE-db/db compared to db/db.

increased, and β-cell mass was 2.1-fold reduced in the db/db mice. In contrast, β-cell apoptosis was reduced, and β-cell mass was partially restored in the db/db IL-1Ra mice (supplemental Fig. 1, *D* and *E*).

PDX1 Is Localized in the Cytosol in T2DM—To investigate whether our results in the two diabetic mouse models can be translated into human diabetes, we analyzed PDX1 localization and expression in human T2DM (Fig. 3, *A–D*) and the effect of IL-1Ra on PDX1 localization in human isolated islets (Fig. 3*F*) and in the rat β-cell line INS-1E (Fig. 3, *G* and *H*). In most β-cells of the control patients investigated in pancreatic sections (Fig. 3, *A* (*a* and *b*) and *B*) or in sections from isolated islets (Fig. 3, *A* (*e* and *f*) and *C*), PDX1 was clearly nuclear (Fig. 3*A*, *b* and *f*). In contrast, PDX1 expression was much weaker in islets from patients with T2DM, and no nuclear PDX1 was observed in (Fig. 3, *A* (*d* and *h*), *B*, and *C*). Similar opposite effects in insulin and PDX1 mRNA expression as observed in the HFD mouse model could be observed in human diabetes. Whereas insulin mRNA in islets from patients with T2DM was reduced to 40% of the expression in islets isolated from non-diabetic control patients, PDX1 was 2.8-fold increased (Fig. 3*D*). This is in

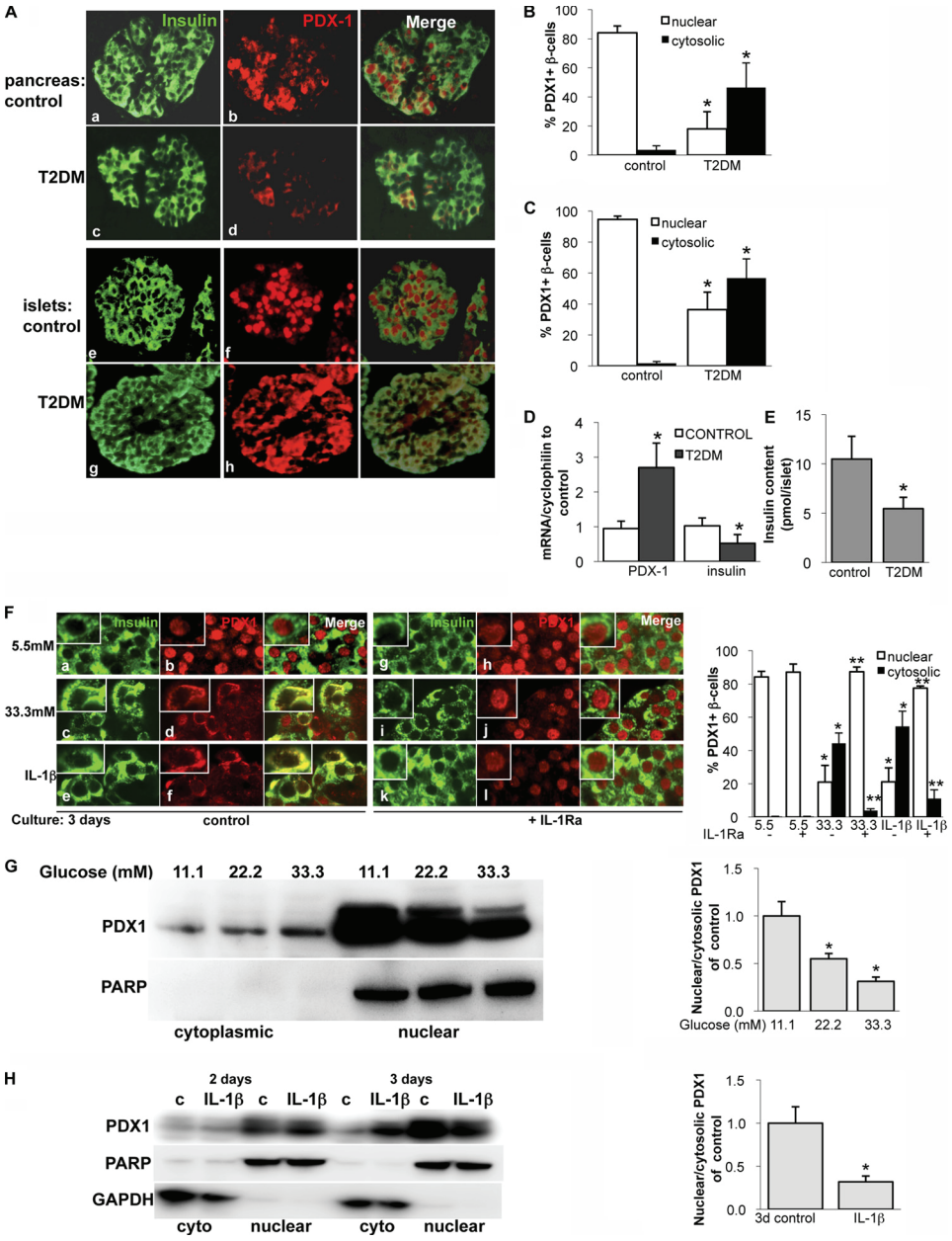

FIGURE 3. **PDX1 is localized in the cytosol in T2DM.** *A*, representative double immunostaining for insulin (*green*; *a*, *c*, *e*, and *g*) and PDX1 (*red*; *b*, *d*, *f*, and *h*) performed in human pancreatic (*a*–*d*) and human isolated islet (*e*–*h*) sections from seven (*c* and *d*) or three (*g* and *h*) poorly controlled patients with T2DM and seven (*a* and *b*) or three (*e* and *f*) healthy controls (magnification, ×250; *inset* magnification, ×2000). The percentage of nuclear and cytosolic PDX1 was calculated by counting 2000 insulin-positive β-cells from seven pancreatic sections (*B*) and three islet sections (*C*) in each condition. *D*, quantitative RT-PCR analysis of insulin and PDX1 expression from mRNA; *E*, insulin content from HCl-ethanol extracts from human islets isolated from control patients and patients with T2DM. In the LightCycler system, mRNA levels was normalized to cyclophilin and tubulin with the same result. Data are shown as mean ± S.E. (*error bars*) from six (mRNA) or three (content) islet isolations from six (three) control patients and six (three) patients with T2DM. *, $p < 0.05$ T2DM compared with controls. *F*, human isolated islets were treated for 72 h with 5.5 mM glucose (control), 33.3 mM glucose, or 2 ng/ml IL-1β with or without 500 ng/ml recombinant human IL-1Ra. Fixed and paraffin-embedded islets sections were double-stained for insulin (*green*; *a*, *c*, *e*, *g*, *i*, and *k*) and PDX1 (*red*; *b*, *d*, *f*, *h*, *j*, and *l*) and analyzed under the confocal microscope. (magnification, ×1000; *inset* magnification, ×4000). Islets were isolated from three different donors, and 3 independent experiments were performed. The percentage of nuclear and cytosolic PDX1 was calculated by counting 2000 insulin-positive β-cells from three experiments in each condition. *G* and *H*, Western blot analysis of PDX1 of glucose-treated (3 days; 11.1–33.3; *G*) and IL-1β-treated (2–3 days; 2 ng/ml; *H*) nuclear and cytosolic cell lysates of the β-cell line INS-1E. GAPDH was used as loading control, and purity of fractions for the cytosolic extracts and full-length PARP for the nuclear extracts in the same membrane after stripping was assessed. One representative blot of three experiments is shown. Densitometry analysis of bands normalized to PARP or GAPDH shows the ratio of nuclear and cytosolic PDX1 expression. Because there were no changes, the 2-day results were not analyzed in *H*.

line with previous data from Del Guerra *et al.* (47). Insulin content from diabetic islets was decreased when compared with islets from control patients (Fig. 3*E*, $p < 0.01$).

Isolated human islets were treated with increasing glucose concentrations (5.5, 11.1, and 33.3 mM glucose) or 5.5 mM glucose plus 2 ng/ml IL-1β for 72 h. As reported before and in our previous studies (4, 48), under conditions of elevated glucose levels or IL-1β treatment, β-cells failed to adequately increase insulin secretion in response to a glucose challenge. As shown in Fig. 3*F* (*b*), PDX1 is localized in the nucleus in insulin-positive β-cells at control conditions at 5.5 mM glucose. Co-exposure of islets to IL-1Ra did not change PDX1 localization (Fig.

3*F* (*h*)). Increasing glucose concentrations to 11.1 (not shown) and 33.3 mM (Fig. 3*F* (*d*)) or the addition of IL-1β (Fig. 3*F* (*f*)) shifted the PDX1 signal to the cytoplasm, resulting in co-localization of PDX1 with insulin (*yellow merged color*), whereas co-incubation with IL-1Ra protected from such PDX1 shift; PDX1 remained in the nucleus in the IL-1Ra-treated islets (Fig. 3*F*, *j* and *l*). In order to show a β-cell-specific mechanism, such changes were confirmed by Western blot analysis of glucose- and IL-1β-treated cell lysates of the β-cell line INS-1E. Glucose induced a dose-dependent decline in nuclear PDX1 together with an increase in cytosolic PDX1 expression after 72 h (Fig. 3*G*). 2 ng/ml IL-1β reduced nuclear and induced cytosolic PDX1 expression after 3 days of exposure but not after 2 days, showing a time-dependent IL-1β effect (Fig. 3*H*).

IL-1Ra Prevents Prolonged Glucose- and IL-1β-induced JNK Activation—PDX1 nuclear export is induced by oxidative stress through a signaling pathway that involves JNK activity (31, 49, 50). Therefore, we investigated whether JNK also mediates glucose- and IL-1β-induced effects on PDX1 localization by first evaluating whether IL-1Ra regulates JNK activation.

In isolated mouse islets, JNK remained in its activated state over a 3-day period in the presence of elevated glucose concentrations (Fig. 4*A*, *left*). In islets from mice that endogenously overexpress IL-1Ra (IL-1Ra-OE), glucose induced transient JNK phosphorylation after 1 day of culture, whereas after 3 days, JNK activation decreased (Fig. 4*A*, *right*). We confirmed these findings in isolated human islets. Phosphorylated JNK levels were increased by a 3-day culture in 33.3 mM glucose or in the presence of 2 ng/ml IL-1β (Fig. 4*B*) compared with control cultures at 5.5 mM glucose. In both cases, this increase was ameliorated when IL-1Ra was added to the culture medium.

Because we postulated that glucose-induced JNK activation leads to PDX1 export, JNK inhibition should prevent shuttling despite the presence of elevated glucose or IL-1β. To test this hypothesis, we treated human islets with 22.2 mM glucose or 2 ng/ml IL-1β, with or without the addition of 10 μM JNKi, a small peptide that inhibits JNK activity, as described before (51, 52). We again observed glucose- and cytokine-mediated PDX1 translocalization (Fig. 4*C*, *top*, *a–c*). We not only found cells that co-express insulin and PDX1 in the cytoplasm but found that some cells displayed PDX1 in the nuclear periphery, which has been observed previously in cell lines kept at low glucose concentrations (34). JNK inhibition prevented glucose- and IL-1β-induced PDX1 translocalization and kept the transcription factor in the nucleus in most cells, indicating that the JNK pathway is involved in regulating PDX1 localization (Fig. 4*C*, *bottom*, *d–f*).

β-Cell apoptosis and function were analyzed in the same experiments. The switch of nuclear to cytosolic PDX1 was accompanied by increased β-cell apoptosis. 3-day incubation of human islets with elevated glucose (22.2 mM) or 2 ng/ml IL-1β resulted in a 1.8- and 2.1-fold increase in β-cell apoptosis, and glucose-stimulated insulin secretion was impaired by 75 and 52%, respectively. In contrast, 1 h of pre-exposure and prolonged culture of the islets with 10 μM JNKi or 250 ng/ml IL-1Ra restored β-cell survival (Fig. 4*D*) and function (Fig. 4, *E* and *F*).

Mutation of the PDX1 Nuclear Export Signal Restores β-Cell Survival and Function—Because we have observed PDX1 nuclear exclusion in a prodiabetic milieu, we tested whether maintaining PDX1 localization in the nucleus may restore β-cell function and survival.

PDX1 WT plasmid and a mutant PDX1, in which the NES-like sequence was disrupted by substituting alanine for leucine at positions 91 and 93 and linked to GFP as described before (33, 49), were expressed in human islets by transient plasmid Lipofectamine-mediated transfection. Plasmids were kindly provided by Dr. Ingo Leibiger. Transfection efficiency was analyzed imunocytochemically by GFP plasmid overexpression, which showed a ~60% transfection efficiency (supplemental Fig. S2). We also evaluated transfection efficiency by Western blot analysis. Linking PDX1 to GFP resulted in an additional band at 70 kDa, which gave an intensive band in the transfected but not in the GFP-transfected islets (Fig. 5*D*). Under these conditions, we analyzed β-cell apoptosis and function. Overexpression of WT or NES mutant PDX1 did not result in significant changes of β-cell apoptosis or function at 5.5 mM glucose. 3-day exposure to 22.2 mM glucose or 2 ng/ml IL-1β resulted in a 1.9- and 2.3-fold increase in β-cell apoptosis and to a 56 and 68% decrease in glucose-stimulated insulin secretion, respectively (Fig. 5, *A–C*, $p > 0.05$), compared with GFP-transfected control. Although WT PDX1 and NES mutant PDX1 improved β-cell survival significantly, glucose-stimulated insulin secretion was only improved by the NES mutant PDX1 and not by WT PDX1 overexpression, indicating 1) that PDX1 overexpression improved β-cell survival and 2) the necessity of nuclear PDX1 for restoration of β-cell function. When PDX1-WT was overexpressed in human islets, IL-1β and glucose induced shuttling of transfected PDX1 to the cytosol (Fig. 5*D*), which resulted in a clear decrease in the nuclear/cytosolic PDX1 expression ratio (Fig. 5*E*). In contrast, PDX1-NES overexpression inhibited such PDX1 shuttling in IL-1β- and glucose-treated islets, and the ratio of nuclear/cytosolic PDX1 expression was significantly increased, compared with PDX1-WT (Fig. 5, *D* and *E*).

Immunostaining of PDX1-NES and PDX1-WT confirmed Western blot results (Fig. 5*F*). PDX1 was localized in the nucleus in insulin-positive β-cells in both WT- and PDX1-NES-transfected islets (Fig. 5*F*, *a*, *b*, *g*, and *h*). Although PDX1-WT expression was shifted to the cytosol in response to IL-1β and glucose treatment (Fig. 5*F*, *c–f*), shuttling-deficient PDX1-NES remained in the nucleus (Fig. 5*F*, *i–l*).

DISCUSSION

Human mutations of PDX1 as well as changes in its transactivation are strongly associated with diabetes (53, 54). In the present study, we show that localization of PDX1 correlates with T2DM, β-cell function, and survival. PDX1 shuttling is one of the mechanisms that may explain the dual role of glucose on β-cell function and survival. Although acute elevated glucose induces β-cell proliferation and insulin secretion, chronically elevated glucose concentrations impair β-cell function, induce apoptosis (40), and thus may accelerate diabetes. Upon acute exposure of β-cells to glucose, PDX1 translocates to the nucleus, leading to insulin gene transcription (34). In contrast,

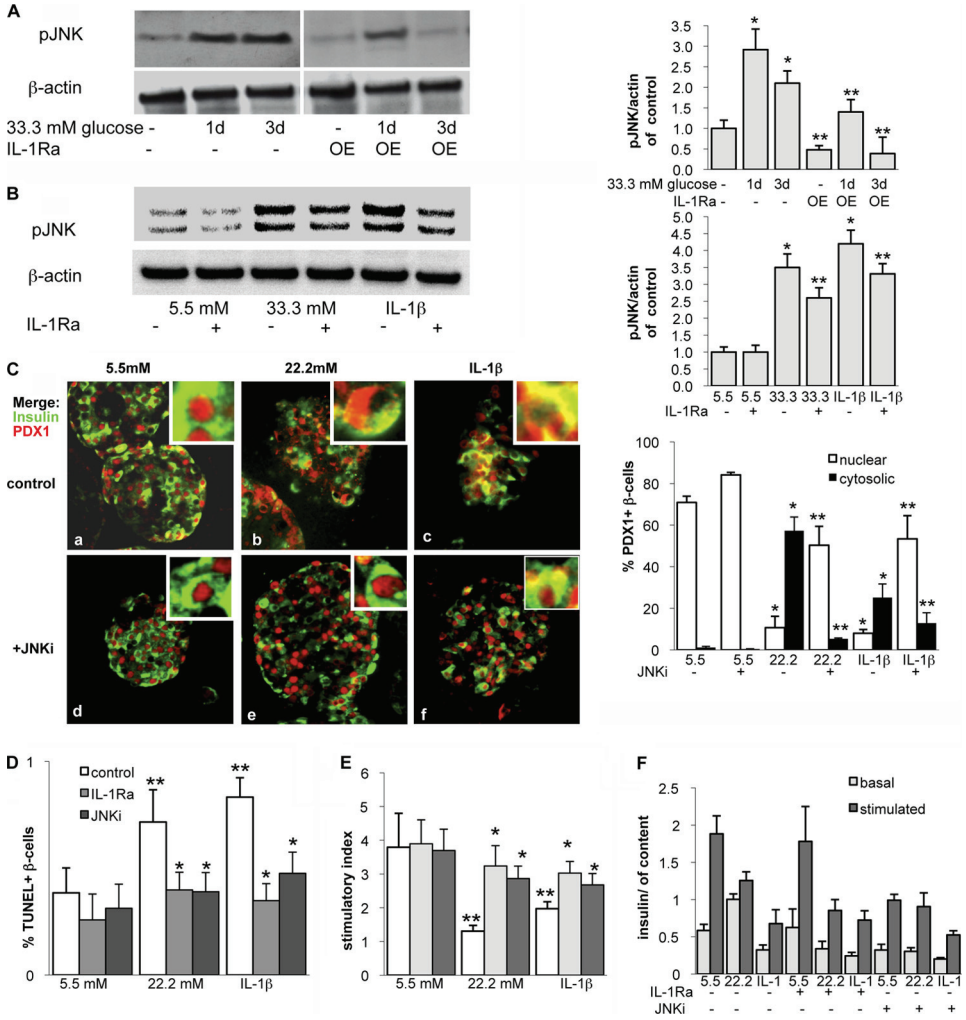

FIGURE 4. **IL-1Ra prevents prolonged glucose- and IL-1β-induced JNK-activation.** *A*, isolated islets from mice overexpressing IL-1Ra (IL-1Ra-OE) and wild type littermates were treated for 24–72 h with 11.1 mM glucose (control) or 33.3 mM glucose. Shown is immunoblotting for phosphorylated JNK and β-actin (loading control). The antibodies were blotted on the same membrane. One representative experiment of three is shown. Shown is densitometry analysis of phospho-JNK bands normalized to β-actin. *B–D*, isolated human islets were cultured in suspension for 24–72 h in 5.5 mM glucose (control), 22.2 or 33.3 mM glucose, or 2 ng/ml IL-1β with or without the addition of 500 ng/ml recombinant human IL-1Ra (*B*) or 10 μM JNKi (*C*). *B*, immunoblotting of phosphorylated JNK and β-actin (loading control). The antibodies were blotted on the same membrane. One representative blot of three experiments from three donors is shown. Shown is densitometry analysis of phospho-JNK bands normalized to β-actin. *C*, after 72 h of treatment, fixed and paraffin-embedded islet sections were double-stained for insulin in *green* and PDX1 in *red* and analyzed under the microscope (magnification, ×250; *inset* magnification, ×2000). Islets were isolated from three different donors, and 3 independent experiments were performed. *D* and *E*, for the analysis of β-cell survival and glucose-stimulated insulin secretion, islets were cultured on extracellular matrix-coated dishes and treated for 72 h. *D*, β-cell apoptosis expressed as a percentage of TUNEL-positive β-cells ± S.E. The mean number of β-cells scored was 2945 ± 218 for each treatment condition in three independent experiments from three different donors. *E* and *F*, glucose-stimulated insulin secretion of islets. Stimulatory index (*E*) denotes the ratio between stimulated (16.7 mM glucose) and basal (2.8 mM glucose) values normalized to insulin content (*F*) of insulin secretion during successive 1-h incubations. Results are means ± S.E. (*error bars*) from three independent experiments from three donors. *, $p < 0.05$ compared with control at 5.5 mM glucose. **, $p < 0.05$, IL-1Ra- or JNKi-treated islets compared with untreated islets under the same conditions.

oxidative stress induces PDX1 shuttling to the cytosol (49) and impairs insulin secretion (55). We show here that in diabetic islets or under conditions of chronic hyperglycemia or IL-1β exposure for 3 days *in vitro*, conditions of impaired β-cell survival and function shifted PDX1 expression to the cytoplasm. These data show the impact of PDX1 localization in regulating β-cell turnover and function.

Whenever it was shifted to the cytosol, PDX1 showed a weaker staining and signal intensity, which suggests the possibility of PDX1 degradation after translocalization. This is also supported by the observation that overexpression of PDX1-NES, which remains in the nucleus, prevents PDX1 shuttling together with degradation at conditions of chronically elevated glucose. Although transfection could not be achieved in all islet cells, nuclear PDX1 overexpression prevented the deleterious effects of glucose and IL-1β on β-cell survival and function. Previously, we have observed such quantitative decrease in PDX1 induced by elevated glucose concentrations in human and rat islets. At this time, whole islet lysates were used for the analysis, and we did not investigate PDX1 localization. PDX1 decrease was dose-dependent on glucose concentrations (5.5–33.3 mM) (56). In the same previous study, we also show an age-dependent PDX1 decrease in human and rat islets, which was confirmed in pancreatic biopsy samples (57). Such PDX1 decrease correlated with the increased susceptibility to glucose-induced apoptosis and with a decline in

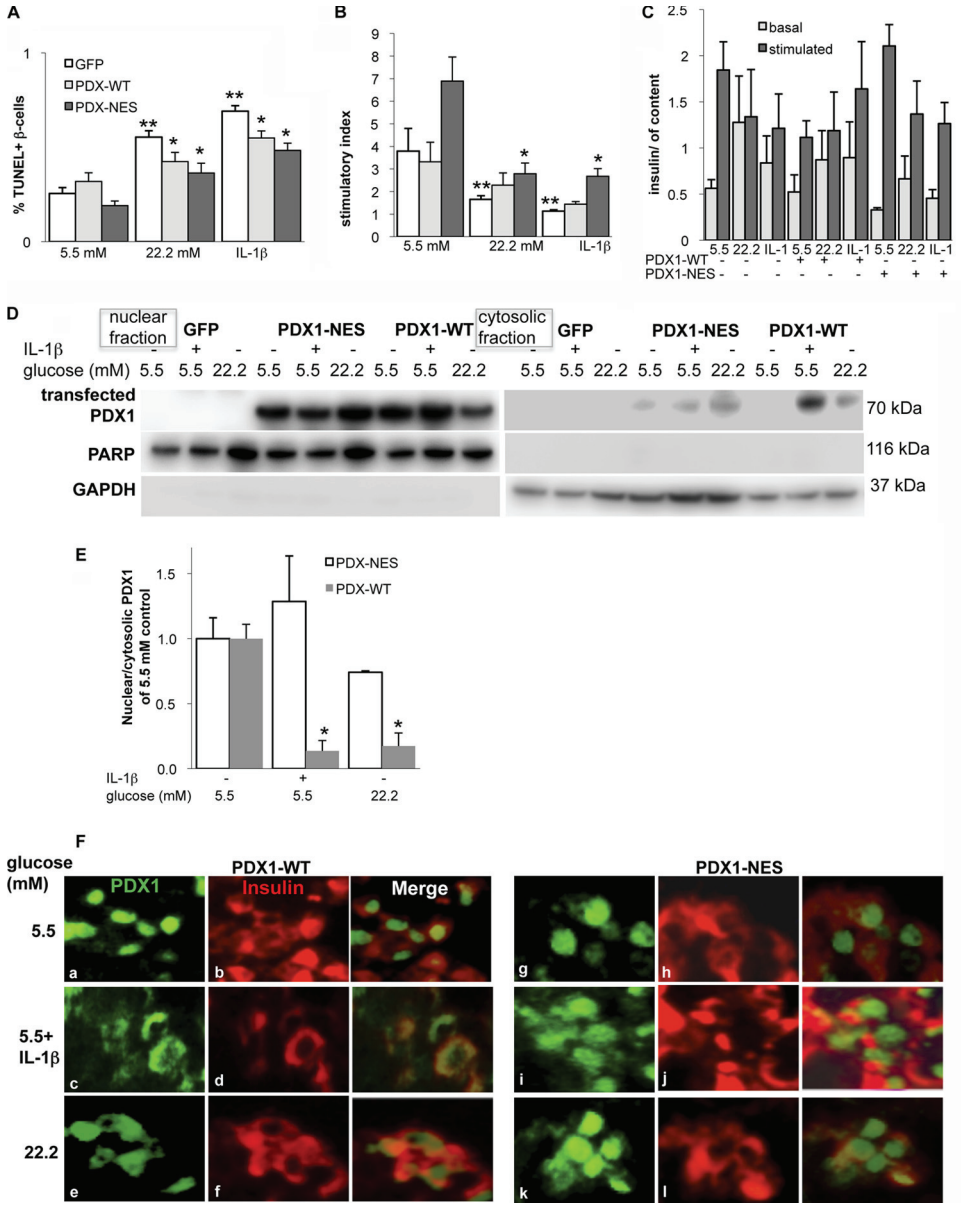

FIGURE 5. **Mutation of the PDX1 nuclear export signal restores β-cell survival and function.** Human isolated islets were cultured on extracellular matrix-coated dishes and transfected with a GFP control plasmid, with WT PDX1, or with an NES mutant PDX1 (PDX1-NES), in which the NES-like sequence was disrupted. 24 h after transfection, islets were exposed to elevated glucose or IL-1β for 72 h. *A*, β-cell apoptosis expressed as a percentage of TUNEL-positive β-cells ± S.E. The mean number of β-cells scored was 1545 ± 112 for each treatment condition in three independent experiments from three different donors. *B* and *C*, glucose-stimulated insulin secretion of islets. *Stimulatory index* (*B*) denotes the ratio between stimulated (16.7 mM glucose) and basal (2.8 mM glucose) values normalized to insulin content (*C*) of insulin secretion during successive 1-h incubations. Results are means ± S.E. from three independent experiments from three donors. **, $p < 0.05$ compared with control at 5.5 mM glucose. *, $p < 0.05$, PDX1-WT- or PDX1-NES-treated islets compared with untreated islets under the same conditions. *D*, Western blot analysis for transfected PDX1 fused to GFP was performed with nuclear and cytosolic fractions of islet lysates. PARP and GAPDH were used as loading control for nuclear (*left*) and cytosolic (*right*) extracts. One representative blot of three experiments is shown. *E*, densitometry analysis of bands normalized to PARP or GAPDH shows the ratio of nuclear and cytosolic transfected PDX1 expression. *, $p < 0.05$ compared with control at 5.5 mM glucose. Islets were isolated from three different donors, and 3 independent experiments were performed. *F*, fixed and paraffin-embedded islet sections were double-stained for PDX1 (*green*; *a*, *c*, and *e* and *g*, *i*, and *k*) and insulin (*red*; *b*, *d*, and *f* and *h*, *j*, and *l*) and analyzed under the fluorescent microscope (magnification, ×800).

β-cell proliferation at an older age. Neither Reers *et al.* (57) nor we were able to show nuclear PDX1 localization. It was suggested that the human pancreas embedding method prevented detection of nuclear PDX1. Here we improved tissue permeabilization and clearly detected nuclear PDX1 even in sections from human autopsy and biopsy, with strong PDX1 signals in the nucleus in non-diabetic control pancreases and a shift of PDX1 to the cytosol in diabetic conditions, a signal that appeared much weaker in sections from patients with T2DM. Together, the data support PDX1 shuttling in response to chronic glucose to the cytosol and its subsequent degradation as one deleterious factor contributing to β-cell failure.

Strategies to block these deleterious effects on the β-cell are needed for a successful diabetes therapy. Blocking IL-1β signals has been suggested as a novel treatment for diabetes. The anti-inflammatory cytokine IL-1Ra prevents glucose-induced apoptosis by blocking proapoptotic IL-1β signaling *in vitro* (4) and

improves glycemia and β-cell function and survival *in vivo* (16, 17, 20).

In this study, we provide further mechanisms of the protective effect of IL-1Ra directly on the β-cell transcriptional regulation in C57BL/6J mice fed a high fat/high sucrose diet (Surwit) and in db/db mice, serving as two animal models of T2DM and in human islets exposed to a diabetic milieu. 12 weeks of high fat feeding induced impaired glucose tolerance, which was inhibited in the IL-1Ra-OE mice. Unexpectedly, insulin and PDX1 mRNA levels were oppositely regulated in the HFD-treated mice; insulin was reduced, and PDX1 was significantly increased in islets after 12 weeks of high fat/high sucrose diet. IL-1Ra prevented such changes. Many previous studies have observed no changes or increases in insulin mRNA in response to a high fat diet without increase in sucrose ("Western diet") in rat or mouse models (*e.g.* see Refs. 58 and 59). The addition of sucrose to the diet (Surwit) causes β-cell failure together with reduced insulin mRNA in the β-cell (60).

Although our results are in contrast to previous data showing PDX1 down-regulation in response to hyperglycemia in β-cells in culture as well as in type 2 diabetic animal models (*e.g.* in ZDF rats (61), in *P. obesus* (29), and in partially pancreatectomized rats (62)), studies in isolated human islets from patients with T2DM show a similar opposite regulation, with reduced insulin mRNA and increased PDX1 mRNA levels compared with islets isolated from non-diabetic controls (47).

PDX1 expression seems to be important for the β-cell response to a higher insulin demand (*e.g.* in insulin resistance and β-cell compensation may occur through increased PDX1 mRNA). But suppression of PDX1 expression in MIN6 cells did not lead to a decrease of insulin or glucokinase mRNA (63). To activate insulin transcription, PDX1 translocates into the nucleus, where it binds to the insulin gene (46, 64). Therefore, post-translational changes, which define PDX1 localization, rather than mRNA expression levels may play a more important role under diabetic conditions. For instance, oxidative stress induces PDX1 shuttling from the nucleus to the cytosol and thus causes a severe reduction of PDX1 activity (49). Also, 24-h exposure of rat islets to palmitic acid at elevated glucose concentrations causes PDX1 localization to the cytosol together with a decrease in MafA expression and inhibition of insulin expression (36). In line with these previous observations, we detected PDX1 predominantly expressed in the cytosol in HFD-treated and in hyperglycemic db/db mice. In contrast, in IL-1Ra-treated as well as the normal diet groups, PDX1 was localized in the nucleus. Because IL-1Ra protected from the prodiabetic effect of the diet, we propose that this is a result of maintaining PDX1 functionally in the nucleus.

Homozygous $Lepr^{db/db}$ mice (db/db) on the C57BLKS/J background with this depletion in the leptin receptor become obese, hyperglycemic, and hyperinsulinemic within the first month of age. In these mice, IL-1β-mediated innate immunity is augmented, which results from a diabetes-associated loss of IL-1β counterregulation (65). To test the hypothesis that IL-1Ra would prevent diabetes progression in this model of T2DM, we injected db/db mice daily with IL-1Ra or with vehicle from 4 weeks of age on. db/db mice that received IL-1Ra showed improved glucose tolerance during intraperitoneal glucose tolerance test experiments after 2 weeks of treatment as compared with their vehicle-treated littermates ($p < 0.05$ at time points 30 and 60 min; data not shown). From 3 weeks of treatment on, we did not observe any differences in glucose levels between the two groups anymore. Considering the short half-life of IL-1Ra (6–8 h) and the 10–100-fold excess that is needed to block IL-1β-mediated effects (66), we tested whether constitutive endogenous overexpression of IL-1Ra would improve the outcome of elevated IL-1Ra levels in db/db mice. We could confirm in the db/db mouse model that IL-1Ra overexpression was protective against the development of diabetes and β-cell failure, although the effects of IL-1Ra in db/db mice were quite modest. However, considering the db/db mouse as a model of severe diabetes with rapid development of hyperglycemia, any significant rescue in the model and a combined effect on glucose tolerance, insulin secretion, β-cell mass, and apoptosis confirms a protective effect on the β-cell. This was paralleled by IL-1Ra-induced PDX1 stabilization in the nucleus in the db/db mice. Already at 6 weeks of age, PDX1 was predominantly expressed in the cytosol in the db/db mice, whereas at the age of 10 weeks, PDX1 was strongly decreased. IL-1Ra restored nuclear PDX1 in 6-week-old mice, and also in 10-week-old mice, nuclear PDX1 expression could be detected. Previous cytochemistry analyses of db/db mouse pancreases show a nuclear loss of MafA rather than of PDX1 (67). To exclude differences in the background, we also analyzed PDX1 in male BKSdb/db mice and could confirm the switch of PDX1 localization to the nucleus already after 6 weeks of age.

Activation of JNK is a hallmark in glucose and IL-1β effect on the β-cell and is involved in PDX1 regulation (68, 69). We used such well known results to confirm our study design and could identify JNK as cellular component also involved in glucose- and IL-1β-regulated PDX1 localization. In wild type islets, JNK was phosphorylated after short term (30 min (data not shown) and 1 day) as well as long term (3 days) incubations with elevated glucose. Therefore, it seems that acute JNK activation does not necessarily cause impairment in β-cell function and survival, whereas chronic activation correlates with glucotoxicity. IL-1Ra protected islets from prolonged JNK activation. JNK activity has previously been linked to PDX1 shuttling under conditions of oxidative stress (49) and in prostaglandin E2-induced β-cell dysfunction (32), together with Foxo1 as a key player (31). Foxo1 cellular localization determines PDX1 localization, and both are reversely expressed. Foxo1 itself is regulated by JNK and AKT activity; JNK induces Foxo1 nuclear import, which leads to PDX1 export, whereas AKT-mediated Foxo1 phosphorylation results in Foxo1 cytoplasmic and PDX1 nuclear localization. Our findings suggest the JNK-PDX1 pathway as a critical signaling network that transduces short term as well as long term glucose stimulation and therefore might partly mediate the dual effect of glucose on β-cell function and survival.

The fact that IL-1Ra potentially prevented hyperglycemia and improved β-cell function favors the critical role of IL-1β signaling in the β-cell not only in a type 1 but also in a type 2 diabetic environment. Our data provide new insights into mechanisms of the protective effect of IL-1Ra on β-cell function and turnover, establish the important role of nuclear PDX1

localization, and support IL-1Ra as a potential therapy for diabetes.

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REFERENCES

1. Donath, M. Y., Schumann, D. M., Faulenbach, M., Ellingsgaard, H., Perren, A., and Ehses, J. A. (2008) *Diabetes Care* **31,** Suppl. 2, S161–S164
2. Ehses, J. A., Böni-Schnetzler, M., Faulenbach, M., and Donath, M. Y. (2008) *Biochem. Soc. Trans.* **36,** 340–342
3. Heitmeier, M. R., Arnush, M., Scarim, A. L., and Corbett, J. A. (2001) *J. Biol. Chem.* **276,** 11151–11158
4. Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H. I., Spinas, G. A., Kaiser, N., Halban, P. A., and Donath, M. Y. (2002) *J. Clin. Invest.* **110,** 851–860
5. Böni-Schnetzler, M., Thorne, J., Parnaud, G., Marselli, L., Ehses, J. A., Kerr-Conte, J., Pattou, F., Halban, P. A., Weir, G. C., and Donath, M. Y. (2008) *J. Clin. Endocrinol. Metab.* **93,** 4065–4074
6. Zhou, R., Tardivel, A., Thorens, B., Choi, I., and Tschopp, J. (2010) *Nat. Immunol.* **11,** 136–140
7. Böni-Schnetzler, M., Boller, S., Debray, S., Bouzakri, K., Meier, D. T., Prazak, R., Kerr-Conte, J., Pattou, F., Ehses, J. A., Schuit, F. C., and Donath, M. Y. (2009) *Endocrinology* **150,** 5218–5229
8. Donath, M. Y., and Böni-Schnetzler, M. (2010) *Cell Metab.* **12,** 427–428
9. Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Tannahill, G. M., Sharp, F. A., Becker, C., Franchi, L., Yoshihara, E., Chen, Z., Mullooly, N., Mielke, L. A., Harris, J., Coll, R. C., Mills, K. H., Mok, K. H., Newsholme, P., Nuñez, G., Yodoi, J., Kahn, S. E., Lavelle, E. C., and O'Neill, L. A. (2010) *Nat. Immunol.* **11,** 897–904
10. Mandrup-Poulsen, T. (2010) *Nat. Immunol.* **11,** 881–883
11. Schroder, K., Zhou, R., and Tschopp, J. (2010) *Science* **327,** 296–300
12. Parikh, H., Carlsson, E., Chutkow, W. A., Johansson, L. E., Storgaard, H., Poulsen, P., Saxena, R., Ladd, C., Schulze, P. C., Mazzini, M. J., Jensen, C. B., Krook, A., Björnholm, M., Tornqvist, H., Zierath, J. R., Ridderstråle, M., Altshuler, D., Lee, R. T., Vaag, A., Groop, L. C., and Mootha, V. K. (2007) *PLoS Med.* **4,** e158
13. Chen, J., Fontes, G., Saxena, G., Poitout, V., and Shalev, A. (2010) *Diabetes* **59,** 440–447
14. Cnop, M., Welsh, N., Jonas, J. C., Jörns, A., Lenzen, S., and Eizirik, D. L. (2005) *Diabetes* **54,** Suppl. 2, S97–S107
15. Igoillo-Esteve, M., Marselli, L., Cunha, D. A., Ladrière, L., Ortis, F., Grieco, F. A., Dotta, F., Weir, G. C., Marchetti, P., Eizirik, D. L., and Cnop, M. (2010) *Diabetologia* **53,** 1395–1405
16. Sauter, N. S., Schulthess, F. T., Galasso, R., Castellani, L. W., and Maedler, K. (2008) *Endocrinology* **149,** 2208–2218
17. Ehses, J. A., Lacraz, G., Giroix, M. H., Schmidlin, F., Coulaud, J., Kassis, N., Irminger, J. C., Kergoat, M., Portha, B., Homo-Delarche, F., and Donath, M. Y. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 13998–14003
18. Osborn, O., Brownell, S. E., Sanchez-Alavez, M., Salomon, D., Gram, H., and Bartfai, T. (2008) *Cytokine* **44,** 141–148
19. Owyang, A. M., Maedler, K., Gross, L., Yin, J., Esposito, L., Shu, L., Jadhav, J., Domsgen, E., Bergemann, J., Lee, S., and Kantak, S. (2010) *Endocrinology* **151,** 2515–2527
20. Larsen, C. M., Faulenbach, M., Vaag, A., Vølund, A., Ehses, J. A., Seifert, B., Mandrup-Poulsen, T., and Donath, M. Y. (2007) *N. Engl. J. Med.* **356,** 1517–1526
21. Donath, M. Y., Böni-Schnetzler, M., Ellingsgaard, H., Halban, P. A., and Ehses, J. A. (2010) *Trends Endocrinol. Metab.* **21,** 261–267
22. Maedler, K., Dharmadhikari, G., Schumann, D. M., and Storling, J. (2009) *Exp. Opin. Biol. Ther.* **9,** 1177–1188
23. Eizirik, D. L., and Mandrup-Poulsen, T. (2001) *Diabetologia* **44,** 2115–2133
24. Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998) *Genes Dev.* **12,** 1763–1768
25. Brissova, M., Shiota, M., Nicholson, W. E., Gannon, M., Knobel, S. M., Piston, D. W., Wright, C. V., and Powers, A. C. (2002) *J. Biol. Chem.* **277,** 11225–11232
26. Johnson, J. D., Ahmed, N. T., Luciani, D. S., Han, Z., Tran, H., Fujita, J., Misler, S., Edlund, H., and Polonsky, K. S. (2003) *J. Clin. Invest.* **111,** 1147–1160
27. McKinnon, C. M., and Docherty, K. (2001) *Diabetologia* **44,** 1203–1214
28. Stoffers, D. A., Zinkin, N. T., Stanojevic, V., Clarke, W. L., and Habener, J. F. (1997) *Nat. Genet.* **15,** 106–110
29. Leibowitz, G., Ferber, S., Apelqvist, A., Edlund, H., Gross, D. J., Cerasi, E., Melloul, D., and Kaiser, N. (2001) *Diabetes* **50,** 1799–1806
30. Kushner, J. A., Ye, J., Schubert, M., Burks, D. J., Dow, M. A., Flint, C. L., Dutta, S., Wright, C. V., Montminy, M. R., and White, M. F. (2002) *J. Clin. Invest.* **109,** 1193–1201
31. Kawamori, D., Kaneto, H., Nakatani, Y., Matsuoka, T. A., Matsuhisa, M., Hori, M., and Yamasaki, Y. (2006) *J. Biol. Chem.* **281,** 1091–1098
32. Meng, Z., Lv, J., Luo, Y., Lin, Y., Zhu, Y., Nie, J., Yang, T., Sun, Y., and Han, X. (2009) *Endocrinology* **150,** 5284–5293
33. Moede, T., Leibiger, B., Pour, H. G., Berggren, P., and Leibiger, I. B. (1999) *FEBS Lett.* **461,** 229–234
34. Elrick, L. J., and Docherty, K. (2001) *Diabetes* **50,** 2244–2252
35. Macfarlane, W. M., Shepherd, R. M., Cosgrove, K. E., James, R. F., Dunne, M. J., and Docherty, K. (2000) *Diabetes* **49,** 418–423
36. Hagman, D. K., Hays, L. B., Parazzoli, S. D., and Poitout, V. (2005) *J. Biol. Chem.* **280,** 32413–32418
37. Glas, R., Sauter, N. S., Schulthess, F. T., Shu, L., Oberholzer, J., and Maedler, K. (2009) *Diabetologia* **52,** 1579–1588
38. Hirsch, E., Irikura, V. M., Paul, S. M., and Hirsh, D. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11008–11013
39. Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A., and Feinglos, M. N. (1988) *Diabetes* **37,** 1163–1167
40. Maedler, K., Spinas, G. A., Lehmann, R., Sergeev, P., Weber, M., Fontana, A., Kaiser, N., and Donath, M. Y. (2001) *Diabetes* **50,** 1683–1690
41. Oberholzer, J., Triponez, F., Mage, R., Andereggen, E., Bühler, L., Crétin, N., Fournier, B., Goumaz, C., Lou, J., Philippe, J., and Morel, P. (2000) *Transplantation* **69,** 1115–1123
42. Schulthess, F. T., Paroni, F., Sauter, N. S., Shu, L., Ribaux, P., Haataja, L., Strieter, R. M., Oberholzer, J., King, C. C., and Maedler, K. (2009) *Cell Metab.* **9,** 125–139
43. Kaiser, N., Corcos, A. P., Sarel, I., and Cerasi, E. (1991) *Endocrinology* **129,** 2067–2076
44. Yoon, K. H., Ko, S. H., Cho, J. H., Lee, J. M., Ahn, Y. B., Song, K. H., Yoo, S. J., Kang, M. I., Cha, B. Y., Lee, K. W., Son, H. Y., Kang, S. K., Kim, H. S., Lee, I. K., and Bonner-Weir, S. (2003) *J. Clin. Endocrinol. Metab.* **88,** 2300–2308
45. Guillemain, G., Da Silva Xavier, G., Rafiq, I., Leturque, A., and Rutter, G. A. (2004) *Biochem. J.* **378,** 219–227
46. Macfarlane, W. M., McKinnon, C. M., Felton-Edkins, Z. A., Cragg, H., James, R. F., and Docherty, K. (1999) *J. Biol. Chem.* **274,** 1011–1016
47. Del Guerra, S., Lupi, R., Marselli, L., Masini, M., Bugliani, M., Sbrana, S., Torri, S., Pollera, M., Boggi, U., Mosca, F., Del Prato, S., and Marchetti, P. (2005) *Diabetes* **54,** 727–735
48. Mandrup-Poulsen, T., Bendtzen, K., Nielsen, J. H., Bendixen, G., and Nerup, J. (1985) *Allergy* **40,** 424–429
49. Kawamori, D., Kajimoto, Y., Kaneto, H., Umayahara, Y., Fujitani, Y., Miyatsuka, T., Watada, H., Leibiger, I. B., Yamasaki, Y., and Hori, M. (2003) *Diabetes* **52,** 2896–2904

50. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., 3rd, Wright, C. V., White, M. F., Arden, K. C., and Accili, D. (2002) *J. Clin. Invest.* **110,** 1839–1847
51. Maedler, K., Schulthess, F. T., Bielman, C., Berney, T., Bonny, C., Prentki, M., Donath, M. Y., and Roduit, R. (2008) *FASEB J.* **22,** 1905–1913
52. Bonny, C., Oberson, A., Negri, S., Sauser, C., and Schorderet, D. F. (2001) *Diabetes* **50,** 77–82
53. Liu, A., Oliver-Krasinski, J., and Stoffers, D. A. (2006) *FEBS Lett.* **580,** 6701–6706
54. Stoffers, D. A., Stanojevic, V., and Habener, J. F. (1998) *J. Clin. Invest.* **102,** 232–241
55. Robertson, R. P. (2006) *Curr. Opin. Pharmacol.* **6,** 615–619
56. Maedler, K., Schumann, D. M., Schulthess, F., Oberholzer, J., Bosco, D., Berney, T., and Donath, M. Y. (2006) *Diabetes* **55,** 2455–2462
57. Reers, C., Erbel, S., Esposito, I., Schmied, B., Büchler, M. W., Nawroth, P. P., and Ritzel, R. A. (2009) *Eur. J. Endocrinol.* **160,** 185–191
58. Briaud, I., Kelpe, C. L., Johnson, L. M., Tran, P. O., and Poitout, V. (2002) *Diabetes* **51,** 662–668
59. Hansotia, T., Maida, A., Flock, G., Yamada, Y., Tsukiyama, K., Seino, Y., and Drucker, D. J. (2007) *J. Clin. Invest.* **117,** 143–152
60. Mulder, H., Mårtensson, H., Sundler, F., and Ahrén, B. (2000) *Metabolism* **49,** 1518–1522
61. Robertson, R. P., Harmon, J., Tran, P. O., Tanaka, Y., and Takahashi, H. (2003) *Diabetes* **52,** 581–587
62. Zangen, D. H., Bonner-Weir, S., Lee, C. H., Latimer, J. B., Miller, C. P., Habener, J. F., and Weir, G. C. (1997) *Diabetes* **46,** 258–264
63. Kajimoto, Y., Watada, H., Matsuoka, T., Kaneto, H., Fujitani, Y., Miyazaki, J., and Yamasaki, Y. (1997) *J. Clin. Invest.* **100,** 1840–1846
64. Rafiq, I., Kennedy, H. J., and Rutter, G. A. (1998) *J. Biol. Chem.* **273,** 23241–23247
65. O'Connor, J. C., Satpathy, A., Hartman, M. E., Horvath, E. M., Kelley, K. W., Dantzer, R., Johnson, R. W., and Freund, G. G. (2005) *J. Immunol.* **174,** 4991–4997
66. Eizirik, D. L., Tracey, D. E., Bendtzen, K., and Sandler, S. (1991) *Diabetologia* **34,** 445–448
67. Harmon, J. S., Bogdani, M., Parazzoli, S. D., Mak, S. S., Oseid, E. A., Berghmans, M., Leboeuf, R. C., and Robertson, R. P. (2009) *Endocrinology* **150,** 4855–4862
68. Størling, J., Zaitsev, S. V., Kapelioukh, I. L., Karlsen, A. E., Billestrup, N., Berggren, P. O., and Mandrup-Poulsen, T. (2005) *Endocrinology* **146,** 3026–3036
69. Kaneto, H., Matsuoka, T. A., Nakatani, Y., Kawamori, D., Matsuhisa, M., and Yamasaki, Y. (2005) *Curr. Diabetes Rev.* **1,** 65–72

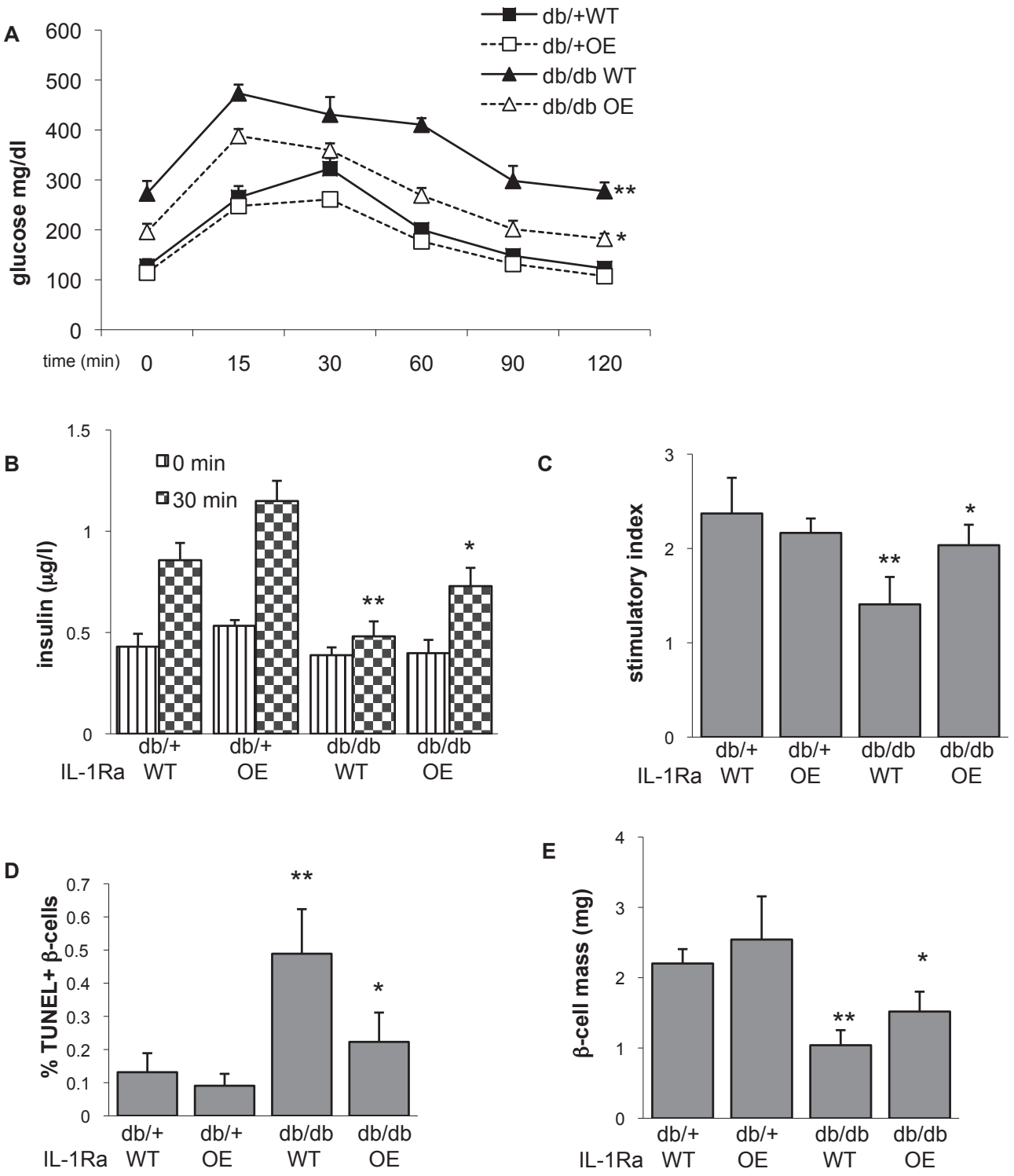

Fig.S1: PDX1 translocalization in db/db mice is paralleled by impaired glycemia and insulin secretion
(A,B) Blood glucose **(A)** and insulin **(B)** levels following i.p. injection of **1 (A) or** 2 **(B)** g/kg body weight glucose in 7-week-old control heterozygous db/$^{+}$, db/$^{+}$IL-1Ra, db/db and db/dbIL-1Ra littermate mice. **(C)** Stimulatory index calculated from stimulated divided by basal insulin secretion **(B)** shows significant decrease in the db/db mice, which was prevented in the db/dbIL-1Ra mice. **(D)** β-cell apoptosis expressed as percentage of TUNEL-positive β-cells ± SE. The mean number of β-cells scored was 2620 ± 256 for each treatment condition in four independent experiments. **(E)** The β-cell mass per pancreas was estimated as the product of the relative cross-sectional area of β-cells (determined by quantification of the cross-sectional area occupied by β-cells divided by the cross-sectional area of total tissue) and the weight of the pancreas. Pancreases were analyzed from 8-week-old mice. **p<0.05 db/db compared to db/+, *p<0.05 db/dbIL-1Ra compared to db/db mice.
Data were collected from 4 animals per group in each of three independent experiments.

Suppl. Figure 1

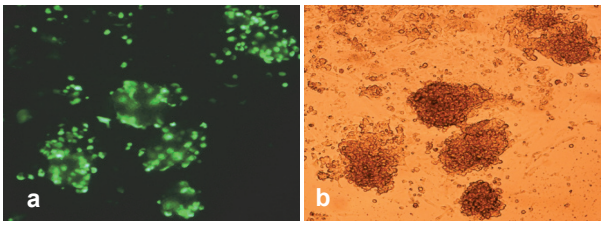

Fig.S2: Transfection efficiency in human islets plated on extracellular matrix coated dishes
Human islets were plated on ECM dishes and transfected with GFP control plasmid (see material and methods). 48 h later, transfection efficiency was determined by fluorescence microscopy (a). (b) shows brightfield picture of all plated cells.

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"*This is the end.. Hold your breath and count to ten..*" With Adele blaring out those words in our lab, I realize that this is really **the end**. As I try hard not be very emotional, I realize how much this journey has affected my life. That's why; this part of the thesis is most important one for me. Speaking for myself, whenever I pick up a thesis, I first check the date of publication (the older the more fascinating, they did some amazing work in the past decades!). Next comes the publication list (Yes Kathrin, I agree it is the most crucial part). But then I quickly start searching for the acknowledgements. I think this section tells me more about the author than any of the previous sections. So, here, I will try to make a good impression without actually losing the genuinity of thought. Readers, be warned. Once I start talking, I cannot stop. This could go on forever, as you might have realized by now, my just introduction of acknowledgements is one paragraph!

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As I pause now for breath, I realize time is running out... need to go through the paper draft again... so I stop here, with a heavy heart. I am sure I missed out mentioning some people in here, but I sincerely want to thank everybody that touched my life here in Bremen. Thanks Guys!! Cheers!!