# 1

# General introduction and outline of this thesis

Type 1 diabetes mellitus (T1DM) is a chronic disease caused by autoimmune mediated destruction of insulin producing beta-cells in the islets of Langerhans in the pancreas. It is the second most prevalent disease among children in the Western world. In the Netherlands, approximately 100,000-120,000 persons suffer from T1DM [1]. There has been a rise in childhood incidence worldwide during the second half of the 20$^{th}$ century attributed to changing environmental factors such as diet, increased hygiene and (entero)viral infections [2]. Currently there is no cure; treatment consists of frequent or continuous insulin administration to mimic beta-cell function. Despite intensive insulin regimes, T1DM still contributes to substantial morbidity and mortality. A significant fraction of patients with T1DM at some point during the course of their disease develop the life-threatening condition of either severe hypoglycemia or ketoacidosis, a state of uncontrolled catabolism due to insulin deficiency. Ketoacidosis can also be the first presentation of T1DM. In spite of insulin replacement therapy, the majority of T1DM patients will eventually develop chronic micro- and macrovascular complications such as nephropathy, retinopathy, peripheral neuropathy, myocardial infarction, stroke and peripheral vascular occlusions. T1DM therefore is associated with severe reduction of quality of life and significant health expenses.

Over the last decades, much progress has been made in our understanding of the pathophysiology and possible starting points of treatment of T1DM. But in spite of this progress, many questions remain unanswered. We know that T1DM is a T-cell mediated auto-immune disease, but the prerequisites for T-cell recruitment to the endogenous pancreatic islets are not fully understood. Specifically, it is unknown whether antigen presence is a prerequisite for T-cell recruitment. The first part of this thesis addresses beta-cell destruction. There is evidence that functional beta-cell mass can be restored after successful immune intervention in diabetes rodent models but we do not know the source of new insulin producing cells nor the role of ongoing autoimmunity in this process. The second part of this thesis addresses beta-cell regeneration. Clinical islet transplantation is an accepted treatment in a subset of T1DM patients. Yet, little is known about T-cell recruitment to islet transplants either. Furthermore there is a growing interest in the contribution of memory T-cells to recurrent autoimmunity in graft failure. T-cell antigen recognition and beta-cell destruction pathways have been largely unraveled, but it is unclear whether we can protect beta-cells by compromising these processes simultaneously, hence whether we can evade the immune system. The third part of this thesis addresses aspects of beta-cell replacement. Clinical trials have shown that anti-CD3 antibodies can temporarily preserve beta-cell function in recent onset T1DM patients but it is not known how safe this treatment is in terms recall immunity (the immune reaction towards pathogens to which patients have been exposed) and preservation of desired immunity against tumours. The fourth and final part of this thesis addresses aspects of immune intervention.

A major rodent model in T1DM research is the non-obese diabetes (NOD) mouse, originally developed in Japan during the selection of a cataract-prone strain. The NOD

mouse spontaneously develops an autoimmune form of diabetes sharing genetic and immuno-pathological features with human T1DM [3]. Yet, despite the similarities between mice and men in the development of autoimmune diabetes, there are many more differences, emphasizing prudence in translating results. Since accessibility to human pancreases during the course of the disease is limited and because reliable biomarkers of both disease activity and aspects of intervention such as efficacy and safety are not readily available, for many studies we currently have to rely on animal models.

**Beta-cell destruction**

T1DM results from a T-cell mediated autoimmune destruction of insulin-producing beta-cells in the islets of Langerhans in the pancreas, in genetically predisposed individuals [4]. Our immune system is programmed to react against non-self antigens: our protection to invaders from the outside world, such as viruses and bacteria. Through negative selection in the thymus, T-cells that react with *self* antigen are deleted, thereby preventing autoimmunity. In T1DM patients autoreactive T-cells escape this central tolerance for reasons not yet completely understood but among which post-translational modification is under a lot of attention [5]. Subsequent exposure of beta-cell proteins (e.g., proinsulin, IA-2, GAD65) to the immune system by for instance local stress or a viral attack, leads to priming and activating of naive autoreactive T-cells. These activated T-cells destruct more beta-cells, with additional exposure of beta-cell proteins to the immune system which continues to fuel beta-cell killing [4]. Why T1DM patients lose tolerance to islet antigens and why the immune system fails to suppress islet autoreactivity is not yet known. Furthermore, neither the nature and function of the different T-cells involved nor the prerequisites for T-cell recruitment are fully determined. In T1DM research, main focus has been on the role of CD4+ T-cells. Upon antigen encounter, such as beta-cell proteins presented by antigen presenting cells (APCs), CD4+T-cells can direct immune responses by differentiation into either effector or regulatory T-cells, depending on the context upon which these are primed [6]. It has become evident however, that CD8+ T-cells significantly contribute to the actual destruction of beta-cells. The pancreatic islets of recent onset T1DM patients show large CD8+ T-cell infiltrates and increased MHC-I expression, molecules necessary for antigen presentation to CD8+ T-cells [7,8]. More recently, specific beta-cell autoreactive CD8+ T-cells could be demonstrated within insulitic lesions of T1DM patients. This indicates that beta-cell destruction involves selective infiltration of autoreactive T-cells [9]. But how selective is this infiltration actually? Studies in a number of infection and autoimmune disease models have suggested that recruitment of T-cells into a site of extra lymphoid inflammation such as the pancreas does not require local expression of cognate peptide-MHC (pMHC) on tissue cells. Or put differently: the fact that T-cells are programmed to recognize a specific antigen does not necessarily imply that these cells need to recognize this antigen in order to infiltrate insulitic lesions. Alternatively, non-antigen specific cues such as cytokines and chemokines may recruit (bystander) T-cells [10,11].

A significant fraction of the islet associated CD8+ T-cells in NOD mice target the diabetogenic epitope *islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)*$_{206\text{-}214}$ [12]. This IGRP epitope is shared between mice and men, and is a target of human diabetogenic T-cells [13,14]. In ***Chapter 2*** we monitored the recruitment of CD8+ T-cells specific for this epitope in gene targeted non-obese diabetic (NOD) mice expressing a T cell 'invisible' IGRP$_{206\text{-}214}$ epitope. The aim of our study was to ascertain whether local expression of cognate pMHC is a prerequisite for the recruitment to and/ or accumulation of CD8+ T-cells in the pancreas during the development of spontaneous autoimmune diabetes. As an alternative outcome, we assessed whether inflammatory cues themselves are sufficient to attract (bystander) T cells. Simplified: are specific T-cells, such as IGRP$_{206\text{-}214}$ -positive T-cells, still recruited to the pancreas when the antigen or epitope they are programmed for to recognize (IGRP$_{206\text{-}214}$), is invisible for them? Rationale behind this study was to understand the complex process of T-cell recruitment and/ or accumulation to endogenous islets during development of T1DM, which on the long run could bring us a step closer to intervening in this process.

**Beta-cell regeneration**

It is estimated that at diagnose of T1DM, 40-90% of the beta-cells have been destroyed. Among the number of issues that need to be addressed in T1DM research, finding a way to restore the severely compromised beta-cell mass/ function is one of them. There is evidence in both rodents and humans that beta-cells are able to adapt their cell mass to various physiological and pathological conditions [15,16,17,18,19,20,21]. The availability of therapies that decrease insulin requirements and temporarily normalize glycemia in T1DM patients [22,23,24] and that can fully reverse hyperglycemia in newly diagnosed diabetic NOD mice [25], raises the question of how functional beta-cell mass could be restored after successful immune intervention. In addition, the worldwide scarcity of pancreas/ islet allografts as an alternative for restoration of beta-cell mass/ function, fuels the exploration of the beta-cell regenerative capacity [26].

For over a century, both replication of pre-existing beta-cells and islet neogenesis, either via trans-differentiation of non-beta-cells or via differentiation of progenitor/ stem cells [27,28] have been proposed mechanisms of beta-cell regeneration. More recently, beta-cell recovery of exhausted beta-cells has been additionally suggested [29]. In the earliest beta-cell regeneration studies, islet cell regeneration was assessed from a morphological point of view. The development of techniques indicating islet cell proliferation, such as 3-H-thymidine incorporation and BrdU labelling, has been a step forward in addressing this issue in preclinical models [27]. However, the true origin of new insulin-producing cells remains difficult to prove. The technique of cell lineage tracing is based on the inheritable labelling of individual islet cells [30]. In T1DM research this means that the islet cell of interest, for instance the alpha-, beta- or duct cell can be marked in such a way that this mark will be transferred to its progeny. Ideally in cell lineage tracing, labelling occurs in a high percentage of the cells of interest, is specific, reliable, bright and without the need for signal enhancing at the same

time. Up until this thesis, beta-cell regeneration studies have been performed in *non*-autoimmune cell lineage tracing models [19,20,21]. Therefore not only the source of new insulin producing cells after successful immune intervention remains unclear, but also the role of ongoing autoimmunity in this process is unknown.



In ***Chapter 3*** we describe the development of lineage tracing models by generating (inducible) expression of red fluorescent protein (RFP) in pre-existing beta-cells and alpha-cells of gene targeted NOD mice. In NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP mice, insulin producing beta-cells were targeted to express RFP in the absence of a Tetracycline antibiotic in food and drinking water (whereas presence of this antibiotic should prevent this). Beta-cells of NOD.RIPCreER.ROSA-tdRFP mice were targeted to express RFP upon injection with an Estrogen Receptor binder, such as Tamoxifen. Alternatively, glucagon producing alpha-cells were labelled with RFP in NOD.GluCre.ROSA-tdRFP mice. Reliable cell lineage tracing models in mice that spontaneously develop an autoimmune form of diabetes could add a novel tool to beta-cell regeneration studies in T1DM research. Because regenerative pathways and regenerative capacity might differ between species, prudence in translating results from animal models is required. However preclinical studies might give some clues and guidance in to what regenerative pathways may be considered.

**Beta-cell replacement**

Ever since the earliest rodent islet transplantation showed restoration of normoglycemia in diabetic recipients, attempts for clinical use of islet cell transplantation have been undertaken [31,32]. Currently islet transplantation is an accepted therapy for patients with complete insulin deficiency, unstable glycemic control and repeated severe hypoglycemia despite optimal diabetes management and compliance [33]. Two-third of T1DM patients that undergo islet cell transplantation show insulin independency 1 year after receiving their final islet infusion [34]. Unfortunately, graft function decreases over time and 5 years post transplantation 90% of the patients have returned to insulin dependency. The majority however continues to display some degree of islet allograft function with decreased insulin requirements, overall better metabolic control and most importantly less episodes of life-threatening hypoglycemia [35]. Challenges in clinical islet transplantation are manifold: there is a scarcity of donor material, significant islet cell loss occurs during the transplantation procedure and current immune suppressive regimens have significant side effects, including intrinsic beta-cell toxicity [36].

Allogeneic islet and pancreas transplants are derived from a donor with a different genetic make-up than the receiver and are thus subject to immune mediated rejection against which systemic immune suppression is required. The first evidence of ongoing autoimmunity as additional threat to graft survival came from a twin to twin (with identical genetic make-up) pancreas transplantation, which showed recurrence of diabetes after initial reversal of hyperglycemia [37]. Huurman *et al* showed in clinical islet transplantation studies that pre-transplantation autoimmune status is predictive of

clinical outcome [38,39]. Another piece of evidence came from Vendrame *et al*, who described that pancreas transplant derived autoreactive T-cells from T1DM patients that underwent simultaneous pancreas-kidney transplantation were able to destroy human islets transplanted into immune-deficient mice [40].

There is a growing interest in the role of recurrent or ongoing autoimmunity in the outcome of allograft islet transplantation. Yet, similar to T-cell recruitment to endogenous islets, little is known about T-cell recruitment to islet transplants. Specifically, there is an interest in the contribution of memory T-cells to recurrent autoimmunity in graft failure.

In ***Chapter 4*** we monitored the recruitment of the same epitope $IGRP_{206-214}$ CD8+ T-cells as previously described, into epitope competent- or epitope invisible grafts, which were transplanted either into diabetic wild type NOD mice (harboring both naive and memory epitope specific T-cells) or epitope invisible hosts (harboring only naive epitope specific T-cells). The aim of this study was two-fold: first, to ascertain whether local antigen expression is a prerequisite for recruitment of CD8+ T-cells in the setting of syngeneic islet transplantation or rephrased: is T-cell recruitment in an islet transplantation model comparable to T-cell recruitment into endogenous islets? And second, to specifically address the contribution of memory T-cells to islet graft failure due to recurrent or ongoing autoimmunity in this non-immune suppressive syngeneic islet transplantation model. Of note, since the transgenic NOD mice that were used are genetically identical, allo-immunity is not addressed here.

Islet cell transplantation is a promising therapy for type 1 diabetes mellitus but, among many other factors, compromised by recurrent islet autoimmunity. Transplantation of genetically immune protected islets to elude host immune responses could be one approach to improve clinical outcome. Direct cell-cell contact initiated by T-cell receptor recognition of antigenic peptide, presented by MHC class I at the surface of the target cells, appears to be critical for beta-cell destruction in T1DM [9,41,42,43]. Furthermore, perforin gene disruption has shown to delay onset of autoimmune diabetes in NOD mice, pointing to the perforin/ granzyme B pathway as the key effector in beta-cell destruction by cytotoxic T-cells [44,45]. In ***Chapter 5*** we tested whether down-regulation of MHC-I expression (a molecule necessary for antigen presentation to CD8+ T-cells), combined with inhibition of granzyme B activity (an enzyme involved in the actual beta-cell killing) via genetically engineered US2/ Serpin 9 expression, protects human beta-cells from acute recurrent islets autoimmunity both *in vitro* and *in vivo*. Or simplified: if compromising both immune recognition of the transplanted islets and the cytotoxic granzyme pathway, would prevent T-cell recruitment and (subsequent) beta-cell destruction.

In order to assess immune protection, reliable measurement of beta-cell toxicity is required. Beta-cell toxicity measurements however are influenced by the quality of the isolated islet fraction, as islet preparations contain a mixture of cell types including beta-cells, alpha-cells, delta-cells, duct cells, endothelial cells, stem cells and leukocytes. We therefore aimed to develop a new strategy to measure beta-cell toxicity and protection

from auto-reactive T-cell mediated killing. In ***Chapter 5*** we additionally tested whether a destabilized luciferase reporter gene, expressed under the human insulin promoter, allows for a specific beta-cell killing assay *in vitro*.

**Aspects of immune-intervention**

Since the 1980s several immunotherapeutic strategies in T1DM have been explored [46]. Targeted immune therapies, such as anti-CD3 therapy, have shown encouraging results in intervention treatment of T1DM, especially in subgroups. Phase II clinical trials with the anti-CD3 antibodies Otelixizumab en Teplizumab showed preserved beta-cell function for at least 18-24 months, decreased insulin requirements and improved levels of glycated haemoglobin in recent onset T1DM patients [21,22,23]. The mechanism of immune suppression mediated by anti-CD3 antibodies is complex, but is believed to depend largely on TCR internalization or unresponsiveness to antigen re-stimulation [47].

A major safety concern in the use of any immune modulating agent in type 1 diabetes mellitus is how well the immune reaction towards pathogens to which patients have been exposed (recall immunity) and desired tumour immunity are preserved. During the European phase II placebo-controlled Otelixizumab (humanized anti-CD3 antibody; ChAglyCD3) trial in recent onset T1DM patients, 75% of the treated patients showed transient Epstein Barr Virus (EBV) reactivation [48]. Although the number of EBV copies returned to normal levels within 1-3 weeks in all patients, comparable with that observed in individuals following infectious mononucleosis in general, this finding emphasizes the importance of addressing recall immunity. Other concerns of immune modulation in T1DM are recurrence or even fuelling of autoimmunity [49]. In ***Chapter 6*** we tested the proliferative T-cell responses towards common pathogens, towards the human tumour suppressor protein p53 and towards several auto-antigens *in vitro* in a sub cohort of the European phase II placebo-controlled Otelixizumab trial in recent onset T1DM patients. Up until now, no follow-up data about recall, tumour and autoimmunity after treatment with anti-CD3 has been available.

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