7

General discussion and conclusions

Type 1 diabetes mellitus results from a T-cell mediated autoimmune destruction of insulin-producing beta cells in genetically predisposed individuals [1]. 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.

Beta-cell destruction

Clinical autopsy studies of recent onset T1DM patients compellingly indicate that beta-cell destruction involves antigen-specific infiltration of autoreactive CD8+ T-cells into insulitic pancreas lesions [2,3,4]. However, studies in a number of infection and autoimmune disease models have suggested additional (bystander) T-cell recruitment in a *non*-antigen specific manner, for instance via cytokines and chemokines [5,6,7]. Presence of bystander T-cells in inflamed tissue could theoretically influence the course of the disease as cytokine production or T-cell receptor engagement as a result of cross-reactivity may provide additional activation signals [5]. And even if these bystander T-cells do not influence the immune pathogenesis of T1DM, the question whether or not T-cell recruitment is solely antigen-specific or antigen-driven stands out, since such knowledge could have possible implications for future therapeutic strategies. Despite the fact that in vitro-activated bystander T-cell clones can transiently co-migrate with their antigen-specific counterparts and that tissue specific expression of cytokine and/or chemokine transgenes in tissue van trigger bystander T-cell inflammation, at least in animal models where the islet infiltrate is often enormous, previous models do not faithfully mimic the events that take place in spontaneous autoimmune inflammation [8]. Specifically it is unclear whether bystander T-cells can effectively compete with their antigen-specific counterparts in occupying inflammatory space.

In ***Chapter 2*** we describe the generation of a genetically engineered NOD strain expressing a T-cell invisible $IGRP_{206-214}$ epitope in beta-cells. These mice developed insulitis and autoimmune diabetes with the same incidence and kinetics as wildtype NOD mice and displayed indistinguishable thymic and splenic profiles. As opposed to wildtype NOD mice however, the islet-associated T-cells of these pre-diabetic mice did not contain $IGRP_{206-214}$-reactive CD8+ cell, as determined by NRP-V7/K^d tetramer staining, nor did these T-cells produce IFNγ in response to NRP-V7 peptide-pulsed APCs. We did find a significant increase in recruitment of other autoreactive T cell specificities, such as insulin-B_{15-23}-reactive CD8+ cells, that were present at very low precursor frequencies in their wildtype counterparts. Additional adoptive transfer experiments revealed that activation and proliferation of naive $IGRP_{206-214}$-reactive CD8+ cell in pancreatic lymph nodes as well as recruitment to the inflamed islets of pre-diabetic NOD mice expressing the T-cell invisible $IGRP_{206-214}$ epitope was severely impaired. Furthermore, pre-activated $IGRP_{206-214}$ specific cytotoxic T-lymphocytes failed to home to the insulitic lesions of these gene-targeted NOD mice.

These data indicate that $IGRP_{206-214}$-reactive CD8+ T-cells are excluded from insulitic

lesions in the absence of local expression of $IGRP_{206-214,}$ suggesting that T-cell occupation to the inflamed islet space in spontaneous autoimmune diabetes is not due to 'diffusion' from the periphery in response to inflammatory and chemotactic cues, but rather to an active process that involves local recognition of cognate pMHC. Furthermore, these data indicate that initiation and progression of spontaneous T1DM in NOD mice does not require the accumulation of $IGRP_{206-214}$-reactive CD8+ T-cells into pancreatic islets. Our observations challenge the generally held assumption that T cell infiltrates in inflamed extra lymphoid tissues, such as pancreatic islets in diabetes, contain a mixture of both cognate and non-cognate (bystander) T cells. Our findings do not argue against the idea that bystander T-cells can transiently migrate to a site of inflammation non-specifically, but rather strongly argue that in the absence of non-cognate pMHC, non-specifically recruited T-cells cannot effectively compete with cognate T cell specificities for occupation of space.

How does this translate clinically? We demonstrate that antigen presence is important in cytotoxic T-cell recruitment and this finding could be translated into strategies that interfere with T-cell recruitment. Our model shows that removing a major diabetogenic epitope in NOD mice, such as IGRP, will not suffice in preventing diabetes nor will it influence the course of the disease. In our T-cell invisible $IGRP_{206-214}$ epitope model, this was illustrated by the significant increase in recruitment of other autoreactive T-cell specificities such as insulin-B_{15-23} reactive CD8+ T-cells. However, inducing tolerance to antigens via the induction of regulatory T-cells may be a possibility to interfere with T-cell recruitment [9]. Furthermore the importance of antigen presence underscores the importance of memory. Memory T-cells are primed to life-long recognize a specific antigen and have the ability to fuel destruction of cells carrying this antigen at any moment. Strategies that interfere with memory in particular or with (re)migration to pancreatic islets therefore are worthwhile exploring too.

The development of an antigen-specific therapy, selectively targeting pathogenic autoreactive T cells, is viewed by many as the best chance to restore immunological self-tolerance [10]. In NOD mice antigen restoring tolerance involves the generation and expansion of antigen-specific regulatory T-cells. In T1DM patients the aim of antigen-specific therapy is to regulate a single islet antigen which will subsequently regulate ongoing autoimmune responses against other islet antigens, via linked suppression [10].

Antigen-specific prevention studies with oral administration of insulin and injection of islet antigens/ peptides, as well as intervention studies in established T1DM patients, have shown limited, and not always consistent results [10]. However, collectively these studies have been reassuring in terms of safety. Specifically, administrating the autoimmune target does not fuel the course of the disease, as was feared [11]. In a recent published antigen-specific intervention study, T1DM patients within 5 years of their T1DM diagnose received a DNA vaccine (BHT-3021), encoding proinsulin [12]. In preclinical studies, this DNA vaccine was capable of preventing and reversing active insulitis in hyperglycemic

NOD mice. Upon weekly intramuscular injections, C-peptide levels improved at 15 weeks with the 1 mg dose, an effect that waned after discontinuation of DNA vaccination in patients. A reduction in antigen-specific CD8+ T-cells targeting proinsulin only was found, suggesting this gene therapy indeed affects CD8+ T-cells selectively under some conditions in some patients. Clinical relevance of this pilot study remains to be confirmed.

The development of antigen-specific therapies faces many challenges, such as patient selection, timing of intervention and optimizing dosing and administration strategies, yet there is a clear rationale for this type of intervention [10,11]. Our findings that T-cell recruitment to inflammatory sites in T1DM is antigen-specific, could act as argument in favour of further pursuing antigen-specific therapies, suggesting that inhibition of islet entry/retention may have a more significant therapeutic benefit that previously appreciated [13].

Beta-cell regeneration

Several therapies haven been able to temporarily preserve beta-cell function in newly onset diabetic patients and to actually restore normoglycemia in NOD mice [14,15,16,17]. However, the mechanism behind restoration of the functional beta-cell mass, either through replication of pre-existing beta-cells or through neogenesis from progenitor cells or differentiated non-beta-cells, remains unclear and highly controversial [18,19,20,21]. Functional recovery of pre-existing beta-cells by immunological remission and glycemic control is another mechanism that could significantly contribute to restoration of function beta-cell mass, comparable to the temporarily insulin-free honeymoon state in newly insulin-treated T1DM patients. After partial or near-total destruction of the mouse endocrine pancreas in a non-auto-immune environment, regeneration of the beta-cells is seen as to different extends. Nir *et al.* showed beta-cell replication as the main source of new beta-cells after 70-80% chemical beta-cell ablation by diphtheria toxin in a cell lineage tracing model [18]. Their labelling percentage was 30%, requiring statistical assumptions in results interpretation. Xu et al showed that endogenous beta-cell progenitors can be activated in the mouse pancreas after partial duct ligation [19]. Near-total chemical ablation by diphtheria toxin in an alternative cell lineage tracing model however revealed evidence for alpha-cell dedifferentiation [20]. Differences in outcome in these studies may be related to differences in experimental models, labelling percentages and in particular differences concerning the percentage of beta-cell destruction as regenerative stimuli used in different models may be insufficient to trigger a neogenesis pathway. Furthermore, none of these studies were performed in mice developing autoimmune diabetes or having insulitis, hence the role of both ongoing inflammation, autoimmunity and restored tolerance in this process is unknown.

In ***Chapter 3*** we describe the development of cell lineage tracing models in mice that spontaneously develop autoimmune diabetes. The individual transgenes that were introduced to facilitate Red Fluorescent Protein expression, showed not to interfere with diabetes susceptibility. The RFP labelling of pre-existing beta cells in our two different models (NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP and NOD.RIPCreER.ROSA-tdRFP) showed

to be bright, cell type specific and achieved high labelling percentages (93.0% ± 1.3; 94.5% ± 1.3). Expression of RFP in pre-existing alpha cells in NOD.GluCre.ROSA-tdRFP mice was somewhat lower (58.0% ± 6.0).

Reliable cell lineage tracing models might help to distinguish between replication, functional recovery or neogenesis of beta-cells as predominant mechanism behind restoration of the functional beta-cell mass. Bright inheritable labelling of pre-existing islet cells in mice that spontaneously develop an autoimmune form of diabetes and that are successfully treated with immune therapy could help to unravel regeneration mechanisms, especially when combined with Bromodeoxyuridine (BrdU) labelling. This thymidine analogue can be incorporated in the newly-synthesized DNA of replicating cells. Upon successful immune-intervention in either NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP or Tamoxifen-treated NOD.RIPCreER.ROSA-tdRFP mice, finding RFP+/ insulin+/ BrdU+ cells would suggest replicating pre-existing beta-cells as the predominant insulin producing source. RFP+/ insulin+/ BrdU- cells on the contrary would imply recovery of pre-existing beta-cells whereas RFP-/ insulin+ cells would point in the direction of a non-beta-cell source. In addition, finding RFP+/ insulin+ cells in NOD.GluCre.ROSA-tdRFP mice after successful immune intervention would suggest alpha-cells as the predominant source of new beta-cells, whereas RFP-/insulin+ cells would make this unlikely. Our current cell lineage tracing models only enable us to trace beta- and alpha-cells, if non-beta, non-alpha-cells are suggested as predominant source of new beta-cells, additional studies would be required.

We successfully generated cell lineage tracing NOD models with bright and specific labelling of a high percentage of their pre-existing beta- or alpha-cells. As spontaneous development of an autoimmune form of diabetes in these mice was not affected, these models could be used to address the origin of insulin producing cells after immune-intervention in preclinical studies. Furthermore these models could help revealing the role of ongoing autoimmunity on beta-cell regeneration.

Although our model adds a novel preclinical tool in diabetes beta-cell regeneration research, a caveat in clinically translating rodent study findings are the possible differences in regenerative pathways and regeneration capacity between species. For instance, an increase in beta-cell duplication has been consistently observed in rodent pregnancies, a strong physiological stimulus for postnatal beta-cell growth [22]. Although similar findings have been described in older autopsy studies of pregnant women [23], a more recent study suggested formation of new islets as the main source of beta-cell mass increase during pregnancy, as opposed to beta-cell duplication. This was based on the higher number of small islets and single beta-cells that were not associated with islets in pancreata of pregnant women compared to non-pregnant women [24]. A recent study in pregnant NOD mice however, using our NOD.RIP-tTA/tet07-Cre.ROSA-tdRFP cell lineage tracing model, suggests that duplication of pre-existing beta-cells is not the sole source of new beta-cells during pregnancy after all,

as the percentage of labeled beta-cells dropped from 97% prior to pregnancy to 87% at mid-pregnancy, an argument in favour of beta-cell replication and neogenesis being not mutually exclusive [25,26].

Evidence for beta-cell neogenesis in diabetes in humans is scarce and outcomes vary. Remaining beta-cells have been histologically demonstrated in individuals suffering from T1DM, even for as long as 50 years. This emphasizes the heterogeneous course of the disease and could also be an argument in favour of beta-cell regeneration, in whatever form [4, 27]. A prospective phase 1-2 study in recent onset T1DM patients undergoing autologous non-myeloablative hematopoetic stem cell transplantation showed prolonged insulin independency compared to the natural course of the disease [28]. The mechanism behind the increased beta-cell function allegedly involves recovery of pre-existing beta-cells, but this interpretation may not be sufficient to explain remission lasting more than seven years. A recent study in T2DM patients found double positive endocrine cells suggesting beta-cell neogenesis as a compensatory mechanism in newly diagnosed T2DM patients [29]. Although the classical pathophysiology of T2DM clearly differs from T1DM, some connexion in beta-cell inflammation with subsequent apoptosis of beta-cell between the two conditions is being found.

At the same time, no evidence for beta-cell regeneration was found in a study performed on human pancreatic tissue collected from 13 patients who underwent partial (50%) pancreatectomy. Differences in outcome may be related to differences in the percentage of beta-cell destruction, which might be insufficient to trigger a neogenesis pathway. In addition, chronic pancreatic inflammation was the underlying cause in the majority of the patients, which might influence beta-cell regeneration capacity. Another explanation however could be that regeneration capacity and regeneration pathways might differ between mice and men, emphasizing precaution in translation of results. Why use animal models to study beta-cell regeneration? First of all, there is limited accessibility to human pancreases during the course of the disease. And even if pancreas material is obtained, evidence of regeneration will be indirect, as opposed to cell lineage tracing studies. As cell lineage tracing cannot be performed in human beings, animal models have been elected. Furthermore, reliable biomarkers for regenerative pathways currently do not exist. Moreover, currently there is no cure for diabetes in humans and therefore it is unknown what happens to beta-cells when the immune attack is terminated. But despite similarities between mice and men in the development of autoimmune diabetes, these are by far outnumbered by differences. Awareness of model limitations and prudence in translation is therefore in order. However preclinical studies might give some clues and guidance as to what regenerative pathways may be considered.

Identifying the source of insulin producing cells after future successful immune intervention could have significant clinical implications. If pre-existing beta-cells are the only source of restored beta-cell mass, the residual beta-cell mass at the time of intervention is expected to predict the outcome of any immune intervention. However,

if other cells serve as beta-cell precursors, outcome should be independent of residual beta-cell mass [30]. Currently, different strategies are being undertaken to create insulin producing beta-cells from stem cells (either human embryonic stem cells or induced pluripotent stem cells) and from endocrine progenitors [31]. In addition to this, identifying regeneration pathways could eventually result in developing strategies capable of enhancing effectiveness of promising immune therapies.

Beta-cell replacement

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 [32,33]. Challenges in clinical islet transplantation remain manifold: there is 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 [34]. In addition, there is a need to specifically address ongoing islet autoimmunity [35].

In ***Chapter 4*** we tested whether T-cell recruitment in an islet transplantation model is comparable to T-cell recruitment into endogenous islets. We monitored recruitment of CD8+ T-cells reactive to the $IGRP_{206-214}$ epitope into epitope competent- or epitope invisible grafts transplanted either in diabetic wildtype NOD mice (harboring both naive and memory epitope specific T-cells) or epitope invisible hosts (harboring only naive epitope specific T-cells). All four host-donor combinations had development of recurrent diabetes within two weeks, indicating that IGRP contributes to but is dispensable for graft destruction in diabetic epitope competent hosts. Wildtype hosts recruited epitope specific T-cells into epitope competent, but not epitope invisible grafts. In epitope invisible hosts, there was no recruitment of epitope specific T-cells, regardless of donor type.

The "non physiological" lymphatic and vascular anatomy of islet cells grafted under the kidney capsule could conceivably render these permeable to bystander T-cells. We demonstrate however that absence of an auto-antigen in syngeneic extra pancreatic islet grafts in diabetic hosts, renders the grafts 'invisible' to cognate memory (and naive) T-cells. Local auto-antigen expression is a requirement for accumulation of antigen-specific T-cells into islet grafts, comparable to T-cell recruitment to endogenous islets.

We furthermore specifically addressed the contribution of memory T-cell to islet graft failure. Tracking of naive splenic CFSE labelled epitope specific T-cells from (8.3) T-cell transgenic NOD mice in wildtype NOD hosts transplanted with epitope competent- or epitope invisible islets showed vigorous proliferation in the lymph nodes draining epitope competent grafts as opposed to the lymph nodes draining epitope invisible grafts. There were however very few donor 8.3-CD8+ T-cells in both epitope competent and epitope invisible grafts. Therefore we conclude that graft derived IGRP did activate naive epitope specific CD8+ T-cells, but graft destruction by memory T-cells invariably predated their recruitment. Our results indicate that recurrent diabetes in the absence of allo-immunity, is driven by auto reactive T-cells primed during the primary immune response.

Our syngeneic rodent islet transplantation model enables partial unravelling of the complex processes of T-cell recruitment and/ or accumulation in transplanted islets. Our findings that previously primed autoreactive T-cells drive recurrent autoimmunity underscores the importance of developing immune strategies to tackle autoreactive T-cell memory after beta-cell replacement therapy. Indeed, studies in clinical islet transplantation from our group had previously demonstrated that reactivation of memory islet autoreactive T-cells is a paramount hurdle to achieve or preserve insulin-independence in transplanted T1D patients, implying that current immune suppressive strategies remain insufficient to deal this autoimmune memory and point to the need of novel immune suppressive therapies targeting memory T-cells [36,37,38]. We contend that our preclinical model may be of service for validation studies here.

Interfering with memory T-cell recruitment by bio-protecting/ encapsulating transplanted islets, could be another possibility [39]. And perhaps, in parallel to the argument favouring the pursuit of antigen-specific therapies in (recent onset) T1DM, our findings that T-cell recruitment to graft sites in autoimmune diabetes is antigen-specific could lead to pursuing antigen-specific therapies as induction therapy in clinical islet transplantation on the long run. But we are not there yet.

The identification of immune markers as correlates for autoimmunity peri-islet transplantation has been a major step forward. The pre-transplant peripheral frequencies of autoreactive T-cells in diabetic islet recipients proved to be predictive of allograft fate: presence of autoreactive T-cells against one or more autoantigens before transplantation was associated with delayed insulin-independence and lower circulating C-peptide levels during the first year after transplantation. Also, post-transplant increases of auto-reactive T-cell frequencies were associated with loss of graft function, suggesting that recurrent autoimmunity plays a paramount role in the outcome of allograft islet transplantation. [36,37,38]. Furthermore, the amount of transplanted beta-cell mass combined with pre-transplant autoreactivity associates with clinical outcome [37]. This once more emphasizes the role of recurrent autoimmunity in islet transplantation and has possible implications for the selection and treatment of T1DM candidate islet recipients.

Transplantation of genetically immune protected islets could be one approach to improve clinical outcome, by avoiding reactivation of islet-antigen specific memory T-cells [40,41,42,43,44]. In ***Chapter 5*** we show that primary human islet cells can be efficiently transduced by lentiviral vectors. To enhance transduction efficiency, islets were dispersed. These 'pseudo-islets', formed by self-reaggregation, proved to be histological and functional comparable to wildtype islets, as confirmed by insulin secretion upon glucose stimulation. The protective effect of combined compromised immune recognition by down-regulation of MHC-I expression (antigen recognition) and inhibition of the cytotoxic granzyme pathway (beta-cell destruction) was demonstrated in surrogate beta-cells and human primary beta-cells by co-culturing these cells with

cytotoxic T-cells directed against an epitope located in the signal peptide of the pre-pro-insuline (PPI) molecule. These autoreactive T-cells are derived from a recent onset T1DM patient. Insulin release upon glucose stimulation was maintained by immune protected beta-cells as opposed to insulin release in non-protected beta-cells. As *in vivo* proof of concept, immune protected human islets were co-transplanted with patient-derived PPI-directed T-cells under the kidney capsule of mice lacking innate immunity and NK cell activity (NOD.SCID. IL-2R$^{-/-}$; NSG mice). Human insulin release and C-peptide levels were monitored following intra-peritoneal glucose-tolerance tests. In agreement with the *in vitro* results, immune protected cells maintained insulin secretion as opposed to non-protected controls, indicating that genetically engineered US2/Serpin 9 expression does not impact islet viability *in vivo*, but instead protects beta-cells from autoimmune T-cell attack.

Our *in vivo* strategy to measure beta-cell toxicity and protection from autoreactive T-cell mediated killing by inserting a luciferase reporter gene specifically in beta-cells proved successful. A killing assay using the autoreactive T-cells isolated from a recent onset T1DM patient directed against PPI was not affected by the quality of the islet isolate. Thus, we engineered a novel assay to assess specific, auto-immune mediated destruction of primary human beta-cells *in vitro*. This approach facilitates the creation of a screening platform for identification of new compounds that inhibit the interplay between beta-cells and autoreactive T-cells. More specifically, this screening platform could be used for the in vitro testing of both efficacy and toxicity of new immune interventions. Furthermore, we designed a preclinical humanized mouse model to allow assessment of the fate of primary human beta-cells in an autoimmune environment. And finally we showed that lentiviral vectors represent an efficient system for gene transfer into human islet cells that can be subsequently reaggregated into functional pseudo-islets. The latter offers new possibilities for genetic modifications to protect human islet cells against the effect of autoreactive and possibly allo-reactive T-cells. By targeting two molecular pathways (MHC class I synthesis and the perforin cell death pathway) we could reinforce human beta-cells to recurrent autoimmunity.

Immune evasion is a possible strategy worth exploring in improving outcome of clinical islet transplantation, be it still far from clinical application. First of all, allo-immunity has not yet been addressed in our study. Furthermore, technical difficulties such as the scaling up of gene transfer under clinically applicable procedures, the stability of transgene expression and the efficacy of down regulation of different HLA haplotypes as well as the risk of tumour development remain to be addressed before any translational research [45]. Yet, experimental clinical gene transfer studies, using lentiviral vectors are currently being pursued in other fields of medicine, delivering the first encouring clinical proof of principle [46,47].

In order to optimize clinical outcome, simultaneous optimizing various 'influenceables' in islet transplantation will be necessary. Among these are islet graft size, the choice of

the engraftment site, encapsulation of islets to protect from host inflammatory reactions while ensuring sufficient oxygen supply during the revascularization period and as mentioned above, choosing the most appropriate and specific immune suppression strategies [39]. Immune evasion could be a strategy to contribute to clinical outcome.

Aspects of immune-intervention

Targeted immune therapies, such as anti-CD3 therapy, have shown encouraging results in the treatment of T1DM, especially in subgroups [14,15,16]. A major safety concern in the use of any immune modulating agent in T1DM is the preservation of anti-tumour immunity and recall immunity (the immune reaction towards pathogens to which patients have been exposed). In the successful European Phase II Otelixizumab (humanized anti-CD3 antibody; ChAglyCD3) trial in recent onset T1DM patients the chosen antibody dosage was considerably higher than the dosage elected for the Phase III DEFEND1 study, which did not reach its primary endpoint of preserved beta-cell function [48]. The dosage had been reduced for safety reasons: during the Phase II Otelixizumab trial, 75% of the treated patients showed transient and self-limiting EBV reactivation [49]. 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.

In ***Chapter 6*** we demonstrate in a sub cohort of the Phase II placebo-controlled trial with humanized anti-CD3 antibody in recent onset T1DM patients that recall immunity is preserved in spite of high-dose anti-CD3 treatment. Proliferative responses towards common pathogens upon in vitro stimulation with different recall antigens were preserved in anti-CD3 treated patients and were highly similar to those in placebo-treated T1DM patients. An additional concern in the treatment of T1DM is the recurrence of auto-immunity actually caused by immune intervention. Monti *et al* [50] proposed homeostatic expansion of auto reactive T cells in T1DM patients receiving islet allografts under anti-IL-2 receptor antibody induction therapy, followed by low dose tacrolimus and rapamycin maintenance therapy. Homeostatic expansion of auto reactive T-cells could lead to exacerbation of autoimmunity and precipitation of the disease. We showed that T-cell responses towards auto-antigens are not significantly altered after high dose anti-CD3 therapy, which means we did not find evidence for reduced or enhanced and fuelled autoimmunity. Furthermore, the proliferative response upon stimulation with the human suppressor protein p53 was invariably high in both the anti-CD3 and the placebo-treated patients underlining preserved desired anti-tumour immunity in spite of anti-CD3 treatment. This observation is in line with the 48 month clinical follow-up where no lymphoma or other malignancies were observed [15]. Although clinical end points were not met in subsequent Otelixizumab studies testing a much lower dose (Phase III DEFEND1 and 2 studies), it seems premature to disqualify anti-CD3 antibodies as potential therapy is recent onset T1DM. Currently a dose-finding Phase I/ Phase II study to investigate Otelixizumab in new-onset autoimmune T1DM patients is ongoing. We demonstrate in this subcohort of recent onset T1DM patients

treated with Otelixizumab, that recall immunity is preserved in spite of high-dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulatory agent in the treatment of T1DM.

Epicrise

As most PhD students, I started my journey as the 'quest for the Holy Grail' [51]. At the near finish of this thesis I wonder whether there is *one* Holy Grail in T1DM research. I contend that an immune intervention can be successful, but in order for a patient to become insulin independent, beta-cell destruction and restoration of beta-cell function need to be addressed simultaneously. Likewise, there will be no successful beta-cell replacement therapy without the necessary immune protection. Furthermore, there is a need to address safety concerns in the development of any immune therapy, while at the same time one has to be aware of the health risks of diabetes itself with current (often suboptimal) insulin treatment regimes. These quests all interconnect just as the projects in this thesis interconnect.

What do I have to offer to a patient with new onset Diabetes Mellitus Type I that was not there before I started this Ph.D.? I wish I could offer a cure, but unfortunately the diabetes field is not there yet, at least in the vast majority of cases. Instead, we talk about T1DM pathophysiology, insulin therapy and blood glucose self-management, and I explain that despite all efforts, unfortunately treatment targets are not met in the vast majority of patients, underscoring the need for alternative treatments. Next, we talk about changing technologies. We talk about the developing closed loop artificial pancreas, aiming at automatically controlling blood glucose levels by providing the substitute endocrine functionality of a healthy pancreas and the (un)safety aspects involved [52]. We talk about a new device developed to 'scan' your subcutaneous blood glucose levels and philosophize if this device will eventually replace the numerous burdensome fingerpricks currently needed [53]. We talk about the several times a year a cure for diabetes is being found in mice, and how no cure currently exists for most humans, hence how this information should be interpreted with prudence in the clinical situation. At the same time, I explain why we use animal models: to gain knowledge on autoimmune diabetes in general that we cannot obtain otherwise.

And perhaps at subsequent visits, we talk about the projects summarized in this thesis, their results and implications. I explain that we show that T-cell recruitment in both spontaneous autoimmune diabetes and islet transplantation requires presence of a cognate antigen, which could be used as an argument in favour of further pursuing antigen-specific therapies. I explain that we show that recurrent diabetes in an islet transplantation model is driven by memory auto reactive T-cells and that this latter finding has contributed to the present testing of immune suppressive drugs that indeed address recurrent autoimmunity, to improve outcome in clinical islet transplantation. I also explain the many current caveats of clinical islet transplantation and that this therapy is currently only available for a specific subset of patients. I explain that we designed

and tested a novel autoimmune diabetes line tracing model for future testing of the regenerative capacity of islet cells. Furthermore, I explain that we showed that immune evasion protects beta-cells from autoimmune T-cell attack in vivo. I explain that currently different immune evasion techniques, such as islet encapsulation are being tested in the clinic [54,55]. I explain that we show that recall immunity is preserved in spite of high dose anti-CD3 treatment, adding to the safety of high dose anti-CD3 treatment as an immune modulator agent in the treatment of T1DM. I also explain that studies testing a much lower dose of anti-CD3 were terminated early as these failed to meet the clinical endpoints and that we are awaiting the results of a dose finding anti-CD3 study. I explain that any intervention therapy most likely will have side-effects, but underscore that the same applies for plainly having T1DM considering the need for frequent insulin injections, the risk of hypo- and hyperglycemia and the almost certain development of micro- and macrovascular complications in time. If we want to cure diabetes, health risks are unfortunately involved and it is only fair to discuss this.

We talk about the combined Holy Grail in T1DM research: the necessity to halt or elude the immune attack in T1DM in a safe manner, while simultaneously restoring beta cell function. We talk about the fact that we are not there yet, but that the diabetes research field is ever developing in its search for a cure. And as an old Chinese proverb states: "*be not afraid of going slow, be afraid only of standing still*".

Gonnie Alkemade

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7