Immune modulation and monitoring of cell therapy in inflammatory disorders

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General Introduction

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The immune system is an intricate network that protects the body against pathogens and cancer. Inflammation occurs in response to harmful stimuli and is a process involving immune cells and molecular mediators. A meticulous balance is necessary in order to rid the body from infection while minimizing tissue damage from inflammation. In inflammatory or autoimmune disorders, the immune system is disturbed and causes damage to the body's own cells and tissue. This results in a variety of disorders depending on the target organ. Immune modulation therapy tempers the disturbed immune system, but most regimen apply relatively broad immune suppression and should therefore be improved. In this thesis, I focused on type 1 diabetes as autoimmune disease to investigate immunopathology and immune modulation therapy with tolerogenic dendritic cells as well as mesenchymal stromal cell therapy in acute graft-versus-host disease, where donor immune cells attack the host tissue.

1. Type 1 diabetes

Diabetes Mellitus is derived from Greek and Latin and translates to "a large discharge of sweet urine". Diabetes mellitus has been known since antiquity and physicians around the world already recognized the characteristic triad of symptoms: polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (1). However, the cause and treatment of this condition remained a mystery for centuries and affected individuals were short-lived. A turning point took place in the 19th and 20th century with scientists investigating the role of the pancreas and insulin in glucose homeostasis, which lead to the first successful treatment of a diabetic patient with insulin derived from canine pancreas (2).

At the present time, we know that diabetes mellitus is a group of metabolic disorders characterized by persistent hyperglycemia which results from defects in insulin secretion or action. The global prevalence of diabetes is estimated to be 463 million people (9,3%)

in 2019 and is expected to rise to 578 million (10,2%) by 2030 (3) and can be classified into 4 groups: type 1, type 2, gestational and specific types of diabetes due to other causes including monogenic predisposition (4). Type 2 diabetes (T2D), accounting for 90-95% of those with diabetes, is caused by a progressive loss of insulin secretion by β-cells combined with insulin resistance (5). Type 1 diabetes (T1D) is caused by an autoimmune destruction of pancreatic β-cells that produce insulin and accounts for 5-10% of the total cases of diabetes worldwide (5). T1D is the major type of diabetes in children and adolescents and an increasing incidence rate is predicted particularly in young children between 0 and 4 during the next years (6, 7). Patients with T1D are condemned to a lifelong treatment regimen with insulin to regulate their glucose balance and are at risk for developing chronic vascular complications of hyperglycemia. Significant technological advances have been made in T1D management including the introduction of continuous glucose monitoring and insulin pump delivery, improving glycemic control as well as the quality of life of diabetic patients (8, 9). Despite the technological advances, T1D patients remain insulin-dependent as treatments addressing the cause of the disease are still in development and under investigation.

2. Pathogenesis of type 1 diabetes

Autoimmune T1D arises from a failure to maintain immune self-tolerance and the consequential destruction of insulin producing β-cells. The primary risk factor for developing T1D is genetic, in particular some of the human leukocyte antigen class (HLA) II genes are highly associated and contribute 40-50% of the inheritable risk to develop T1D (10). Indeed, either HLA-DR3-DQ2 or HLA-DR4-DQ8 are present in approximately 95% of T1D patients, compared to 50% or less of the general population. It is believed that environmental factors trigger islet autoimmunity in individuals with genetic susceptibility. Potential environmental triggers include infections, microbiota, dietary intake and toxins affecting children in utero or during early childhood (11). Increasing evidence is arising supporting the β-cell stress hypothesis which proposes that factors causing increased insulin demand disturbs β-cell function resulting in a secondary autoimmune response. β-

cell endoplasmic reticulum (ER) stress influences transcription, translation and post-translational events which can lead to generation of neo-antigens to which central tolerance is lacking (12, 13). Peripheral tolerance mechanisms that should prevent autoimmunity fails in T1D patients and an autoimmune cascade is initiated (Figure 1). A gradual loss of β-cell mass results in an initial period of dysglycemia and eventually hyperglycemia due to insufficient endogenous insulin production and more β-cell stress, which is when T1D is typically diagnosed. Interestingly, contrary to claims in text books of medicine, residual β-cell mass can be detected after onset and in T1D patients with long standing disease and basal insulin production is preserved in some patients leaving a window of opportunity for intervention therapies (14). To understand success and failure of future intervention therapies, there is an unmet need for biomarkers and immune correlates of disease progression and therapeutic intervention. In this thesis, I addressed this by extensive phenotyping of immune cell populations involved in T1D and investigating changes in immune responses following intervention therapy.

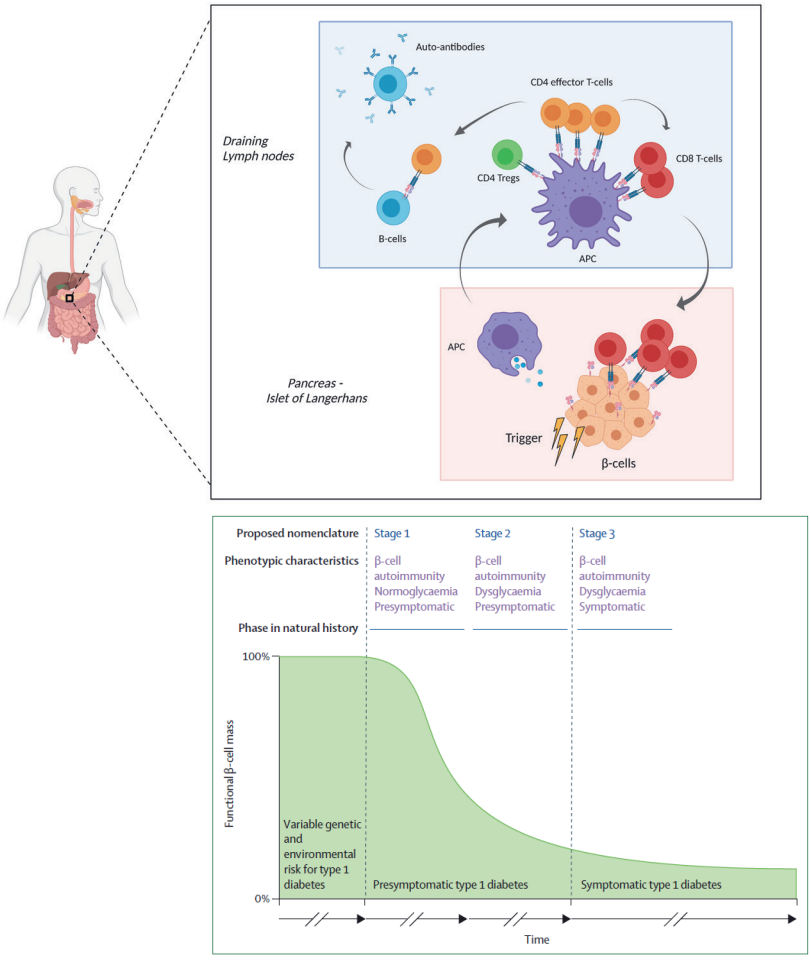

Figure 1. Type 1 diabetes results from an autoimmune mediated attack on insulin producing β-cells in the pancreas. Antigen presenting cells (APC) are alerted and activated due to an unknown trigger, take up β-cell antigens in the pancreas, migrate to draining lymph nodes and present β-cell antigens to T-cells. Naïve CD4 T-cells are primed and activate cytotoxic CD8 T-cells. In addition, CD4 T-cells activate B-cells to produce islet-specific autoantibodies. Regulatory T-cells (Tregs) are unable to keep autoreactive CD4 and CD8 T-cells in check and CD8 T-cells migrate to the pancreas and specifically target β-cells. Different stages of disease reflect the loss of functional β-cell mass. A pre-symptomatic phase precedes symptomatic dysglycaemia where patients are typically diagnosed. Top figure was created in BioRender.com and bottom figure was published in F. Pociot et al, Lancet 2016.

Antigen presenting cells

The autoimmune cascade following eliciting factors likely starts with antigen presenting cells. The strong link between specific HLA class II haplotypes and T1D, points to an essential role of antigen presentation to CD4 T-cells in the disease pathogenesis. HLA-DR3-DQ2 and HLA-DR4-DQ8 contain unique structural properties which select a distinct peptidome, including islet antigens (15). Antigen presenting cells resident in the pancreas are mostly macrophages and to a lesser extent dendritic cells (DCs) (16, 17). DCs and macrophages play important roles in directing innate and adaptive immune responses as well as maintaining tissue homeostasis (18). In animal models, macrophages were key for T1D progression and their depletion resulted in reduced insulitis and islet-peptide presentation to T-cells (19, 20). In humans, macrophages are the second most prevalent immune cell type to be found in the islets of early and late T1D (21). Whether macrophages or DCs are responsible for initiating autoimmunity and to what environmental stimuli they respond is unknown. Dendritic cells but also macrophages could present islet-antigens, migrate to draining lymph nodes and prime and expand islet-reactive T-cells thereby initiating autoimmunity (22). Islet (infiltrating) macrophages could also exhibit cytotoxic effects and directly partake in the destruction of β-cells. Since antigen presenting cells determine the course of adaptive immune responses, it seems interesting to assess whether modulation of these cells towards a tolerogenic phenotype may reduce inflammation and disease. In this thesis, I explored a novel strategy of tolerogenic dendritic cell (tolDC) therapy and investigated the immunological effect *in vitro* and *in vivo.*

T-cells

The pathogenic role of CD4 and CD8 T-cells in T1D is well established. Islet lymphocytic infiltrates are observed in most cases with recent-onset T1D (23), this infiltrate is predominated by CD8 T-cells and accompanied by lower numbers of CD4 T-cells (21). The recruitment of autoreactive CD4 T-cells seem to be required for the initiation of autoreactivity, yet CD8 T-cells are the key pathogenic mediators in the destruction of β-cells (24, 25) and CD8 T-cells specific to islet antigens have been detected in insulitis lesions of T1D patients (26). Autoreactive CD8 T-cells recognize peptides in HLA class I molecules on the surface of β-cells and induce β-cell apoptosis. In addition to strongly associated HLA class II genes, class I HLA-A and HLA-B alleles have been linked to T1D (27). A range of T1D associated HLA I restricted epitopes that can be targeted by autoreactive CD8 T-cells have been identified including GAD, IA2, IGRP, Znt8 and proinsulin (28). Interestingly, particularly CD8 T-cells targeting low affinity binding islet peptides were detected in T1D (29, 30). This explains the presence of these low affine autoreactive T-cells in the circulation, since high-affinity binding autoreactive T-cells are deleted in the thymus by negative selection (central tolerance). On a different note, there is an increasing interest in neo-antigens that arise in response to local β-cell inflammation, which T-cells recognize as foreign proteins (13, 31). One example of such a neo-antigen is the result of an alternative open reading frame within human insulin mRNA encoding a Defective Ribosomal product (DRiP) (32). CD8 T-cells recognizing insulin DRiP were found in the blood of T1D patients and killed β-cells *in vitro*. Because of technological challenges, characteristics of autoreactive and neoantigen-specific CD8 T-cells are scarcely described. In this thesis, I studied these rare cells by combining detection of antigen-specific cells with tetramers and mass cytometry.

Adoptive transfer studies in mice showed that β-cell specific CD8 T-cells are capable of transferring diabetes, but more efficiently in presence of CD4 T-cells (33, 34). Autoreactive CD4 T-cells can be primed by antigen presenting cells and differentiate to Th subsets that can help activate B and cytotoxic T-cells. T1D in this regard is viewed as an IFN-ɣ/Th1-mediated pathology, with autoreactive T-cells exhibiting a polarization towards IFN-ɣ

secretion (35, 36). More recently, the role of Th17 cells in T1D has also been considered. Several studies showed an increase of Th17 cells expressing CCR6 in the circulation of children with recent-onset disease (37) and Th17 cells in pancreatic lymph nodes were still detected in patients with long-term disease (38). Lastly, an increased production of IL-21 by T-cells, with potential pro-inflammatory effects on several immune cells, have been observed in patients with T1D (39). IL-21 is characteristic for follicular helper cells and a similar increase of follicular helper cells expressing CXCR5 was observed in T1D (40). Altogether, it seems that different Th subsets are involved in T1D, including Th1, Th17 and Tfh cells, though it is unclear how these different Th subsets contribute into the inflammatory cascade. It is not surprising that T-cell depleting agents have been investigated as intervention therapy in T1D; these studies showed promising results as beta cell function was preserved in new-onset patients (41, 42). TolDC also show capacity to inhibit effector T-cells *in vitro.* In this thesis, I investigated whether CD4 or CD8 T-cell subsets are affected by tolDC *in vivo.*

Tregs

Regulatory T-cells (Tregs) are a specialized population of T-cells that play an essential role in maintaining peripheral immune tolerance. Tregs are typically characterized by the expression of intracellular FoxP3, high expression of CD25 and the absence of CD127 (43). However, these markers may not include all Tregs since FoxP3 negative Tregs have also been described (44, 45). Conversely, conventional CD4 T-cells can upregulate FoxP3 and CD25 after activation (46), further complicating the characterization of Tregs. Tregs can regulate through direct inhibition via cell-cell contact, secreting anti-inflammatory cytokines such as IL-10 and TGF-β and depriving cytokines from neighboring effector cells (47). Defects in Treg function have been found in patients with autoimmune disease (48), illustrating the crucial role of Tregs in preventing autoimmunity. Though the frequency of $FoxP3^+$ Tregs appears unaltered in T1D patients (49), several studies have pointed to a reduced ability of Tregs to suppress effector T-cells (50, 51). This was attributed to reduced suppressive function of Tregs but also to resistance of effector T-cells for Treg-

mediated suppression, which was varying between individuals (51, 52). Similarly, a decreased fraction of activated Tregs were found in T1D patients (53). The function and generation of Tregs is dependent on IL-2 signaling and polymorphisms in the IL-2 signaling pathway have been associated with T1D (54). Interestingly, a subgroup of T1D patients demonstrated a decreased sensitivity to IL-2 and Tregs from these individuals showed reduced frequency and suppressive function (55). Taken together, it appears that the degree and type of defects in Tregs are heterogeneous among T1D patients. Regardless of the mechanism of action, there seems to be a disbalance between diabetogenic autoreactive T-cells and Tregs which leads to a breach of peripheral tolerance. Restoration or amplification of Tregs is therefore a logical approach to inhibit T1D progression. Indeed, ex-vivo expanded polyclonal Tregs were administered to T1D patients in a phase I clinical trial (56), but clinical effects are yet to be proven. Another approach to amplify Tregs *in vivo* is with tolDC. TolDC *in vitro* induce antigen specific Tregs that suppress through cell-cell contact upon interaction with their cognate antigen (57), but also inhibit autoreactive CD4 and CD8 T-cells. Thus, tolDC may act superior to Treg infusion alone. Together with CD4 Th and CD8 T-cell subsets, I followed change in Tregs after tolDC therapy in T1D patients to establish the immunological efficacy of tolDC therapy.

B-cells and islet autoantibodies

Islet autoantibodies are key biomarkers predicting the development of T1D. Within 20 years, 10% of individuals with 1 autoantibody (AAb) and nearly 100% of individuals with multiple AAb developed T1D (58). Therefore, B-cells and AAb are generally considered to participate in the pathogenesis of T1D. To date however, their exact role is still unclear. B-cells are capable of taking up and processing antigens but only through antigen-specific AAb on their surface, yet their HLA-DR peptidome is different from that of monocyte derived DCs (59). Furthermore, B-cells can differentiate to plasma cells and produce (auto)antibodies. Indicative for a pathogenic role of B-cells is the clinical effect of B-cell depleting agent rituximab, which delayed the loss of beta cell function over a period of 8 months (60). In contrast, a patient with hereditary B-cell deficiency developed T1D,

demonstrating the redundancy of B-cells and AAb for the development of T1D (61). Supporting the latter, therapies attempting to remove circulating islet AAb had negligible effects (62, 63), while transmission of maternal islet AAb protected offspring from developing T1D (64). Moreover, targets from currently known islet AAb locate intracellularly, precluding direct cytolytic activity of β-cells by these AAb. Considering the limited capacity of B-cells to prime T-cells (65), it is unlikely that B-cells play a significant role in the initiation of autoimmunity in T1D. It is more likely that B-cells are activated and differentiating to produce AAb following autoreactive T-cell priming by DCs or macrophages. As a result, the presence of circulating AAb reflects the initiation of autoimmunity in T1D without necessarily exerting a pathogenic role itself.

3. Tolerogenic dendritic cell therapy

As described in the previous paragraphs, T1D arises from an immunological disbalance and involves several immune populations with the CD8 T-cells as ultimate effectors targeting β-cells. Dendritic cells (DCs) act as sentinels of the immune system and have the ability to orchestrate pro- and anti-inflammatory responses. The feasibility of *ex-vivo* generation of DCs from peripheral blood monocytes allows the therapeutic application of DCs. Inflammatory DCs have been used in cancer immunotherapy to boost anti-tumor effects (66). DCs with regulatory capacity, tolDC, can inhibit immune responses and can be induced by several modulating actors, such as vitamin D and dexamethasone (67). Tolerogenic dendritic cell (tolDC) therapy is a promising therapeutic candidate to address T1D at the root of the disease and restore immunological balance. When loaded with autoantigen, tolDC can specifically target autoreactive T-cells leaving immunity against pathogens intact. TolDC therapy is evaluated in several autoimmune diseases with promising results (68, 69), encouraging further investigation in T1D. Considering the antigen-specific effect of tolDC, determining the immunological effect of tolDC *in vivo* is challenging and requires optimization and exploration of novel (antigen-specific) assays to monitor immune modulation. The various mechanism of action of tolDC and methods to monitor immune modulation are reviewed in **Chapter 4.1**.

4. Mesenchymal stromal cell therapy in graft versus host disease

Mesenchymal stromal cells (MSC) are undifferentiated multipotent cells that can be found in most tissues in the body, such as adipose tissue, bone marrow and muscle, as well as pancreatic islets of Langerhans (70, 71). Their capacity to differentiate into various cell types renders MSC attractive as regenerative treatment for various clinical conditions. Besides supporting tissue regeneration, MSC also show immune modulatory capacity *in vitro*. MSC can inhibit T-cell proliferation and induce the expansion of Tregs, similar to tolDC (72-74). Furthermore, MSC can induce M2-like macrophages and inhibit expansion and activation of B-cells and NK cells (75-77). In contrast to the cell-contact dependent regulatory mechanism in tolDC, MSC seem to exert their effect through soluble factors such as TGF-β, IDO and PGE2 (75, 78). Having the capacity to support tissue regeneration and modulate immune responses, MSCs have been applied in the treatment of ulcerative colitis, kidney transplant rejection and steroid-refractory graft versus host disease (GvHD). GvHD is a serious complication following allogeneic hematopoietic stem cell transplantation (aHSCT). Acute GvHD manifests within 100 days after aHSCT and main organs that are affected are the skin, gastrointestinal tract and liver. GvHD occurs when T-cells from the graft recognize the recipient (host) as foreign. An immune response is elicited in which donor T-cells attack recipient cells. MSC treatment in acute GvHD was shown as effective therapy in a subset of patients and ameliorated chances of survival (79). Determining the exact mechanism of action *in vivo* and finding signatures predicting response to MSC therapy will improve future clinical applications with MSC.

5. High-dimensional analysis of immune cells with mass cytometry

Investigating the complexity of the immune system is a challenging task. For decades, immunologists have relied on flow cytometry to classify and characterize immune populations by labelling cells with fluorescent markers. With the arrival of mass cytometry, a relatively novel cytometry platform, more detailed analysis could be performed. In mass cytometry, metal ions with minimal signal overlap instead of fluorophores with broad emission spectra are utilized. This allows the simultaneous detection of more than 35 markers, doubling the range from flow cytometry and enabling identification of novel and rare immune populations (80, 81). The application of mass cytometry is therefore valuable to further our understanding of the role of the immune system in inflammatory disorders and to extensively analyze affected cell populations in immune intervention therapies. In this thesis, mass cytometry was broadly applied to investigate various immune cell populations in T1D and graft-versus-host disease before and after cellular immune intervention therapy.

6. Thesis outline

The main objective of this thesis is to investigate the effect of immune modulatory cell therapy in autoimmune T1D. To gain further insight into pathologic processes underlying T1D, we examined pancreatic sections from an individual with multiple autoantibodies at high risk of developing T1D in **Chapter 2**. We took this rare opportunity to study pathologic changes in the pre-diabetic phase before the complete destruction of β-cells. In **Chapter 3**, we investigated circulating autoreactive CD8 T-cells in patients with established T1D using mass cytometry. With HLA class I tetramers, antigen-specific CD8 T-cells were detected including CD8 T-cells specific for INS-DRip neoantigen. Next, we investigated the regulatory action of tolDC in **Chapter 4**. **Chapter 4.1**. reviews how tolDC can target pathogenic processes occurring in autoimmune diseases such as T1D, but also Rheumatoid Arthritis, which bears much resemblance to T1D. The induction of Tregs is one of the most important mechanism of action of tolDC, though markers delineating induced Tregs, in particular tolDC-induced Tregs, are lacking. Therefore, we extensively analyzed the phenotype of Tregs induced by tolDC *in vitro* using mass cytometry in **Chapter 4.2** to provide means for monitoring Treg induction *in vivo.* Finally in **Chapter 5,** we applied cellular immune intervention therapy in clinical trials and monitor immunological effects *in vivo*. The mechanism of action and potential methods to monitor immunologic effect of tolDC were reviewed in **Chapter 5.1**. In **Chapter 5.2**, we evaluated the effects of intradermally injected tolDC pulsed with proinsulin peptide in a clinical trial with T1D patients. Translating our understanding of immune modulatory cell therapy and analysis with mass cytometry, we investigated treatment with mesenchymal stromal cells in children with steroid-refractory graft versus host disease in **Chapter 5.3**. **Chapter 6** concludes the main findings from this thesis in a summarizing discussion.

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