From immune suppression to immune modulation in type 1 diabetes patients

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Kayleigh Montana van Megen •

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To develop a complete mind:
Study the science of art;
Study the art of science.
Learn how to see.
Realize that everything connects to everything else.

– Leonardo da Vinci (1452-1519)

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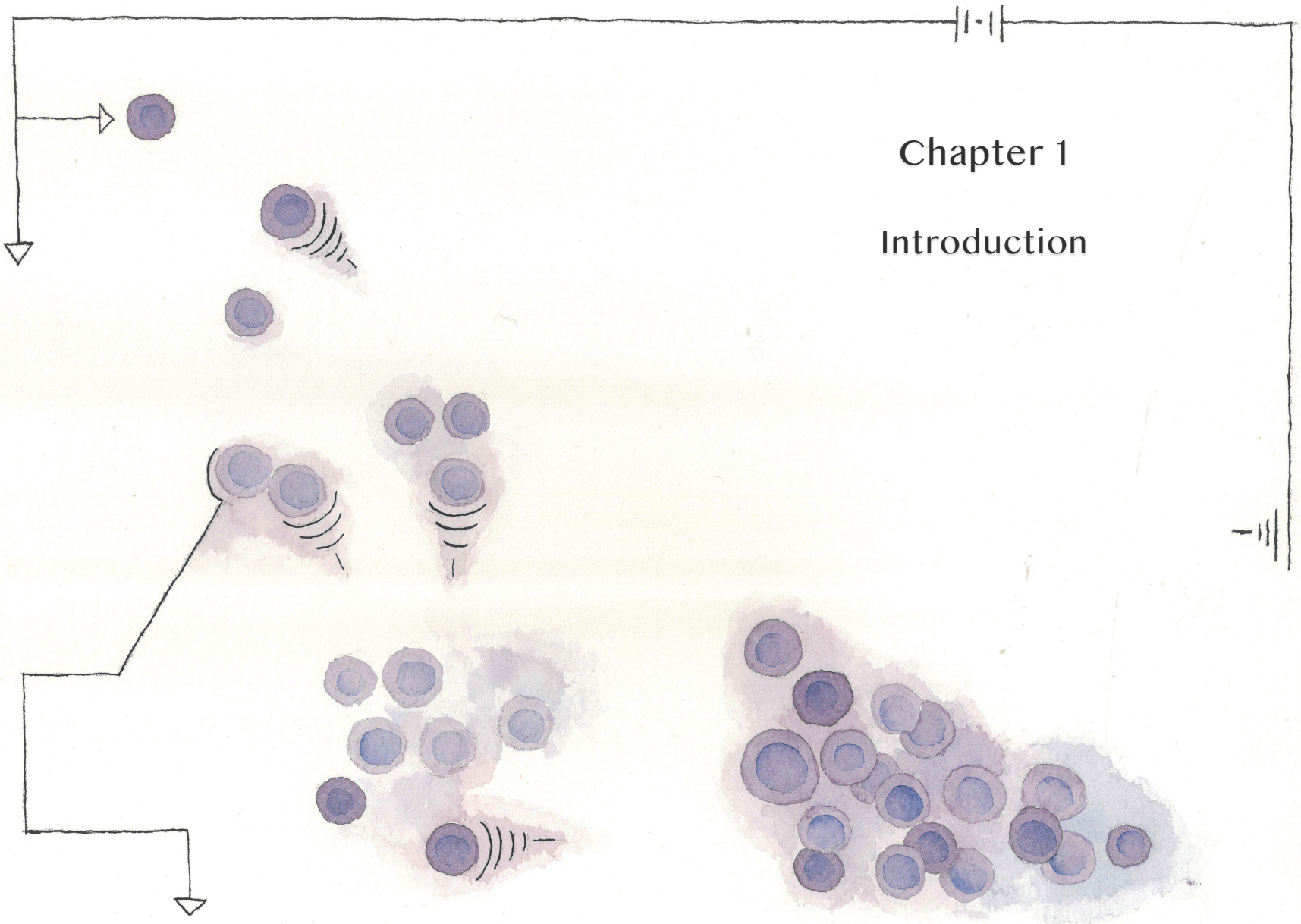

Chapter 1

Introduction

1. Type 1 diabetes

Type 1 diabetes (T1D) is an auto-immune disease characterized by the destruction of the insulin-producing β cells in the pancreas. Insulin is a hormone that lowers blood glucose levels by facilitating the uptake of glucose in peripheral tissues. Therefore, T1D patients present with high blood glucose levels at diagnosis (1).

1.1. Clinical diagnosis of type 1 diabetes

The clinical diagnosis of diabetes is made by various laboratory tests, namely a fasting blood glucose higher than 7 mmol/L (126 mg/dL), symptoms of hyperglycemia with any blood glucose of 11.1 mmol/dL (200 mg/dL) or higher, or a 2 hour oral glucose tolerance test of more than 11.1 mmol/dL. More recently, glycated hemoglobulin (HbA1c) of 6.5% or higher has been added as an independent diagnostic criterion, which reflects glucose control in the previous eight to twelve weeks (2). A new staging system for T1D was proposed in 2015, which allows for diagnosis before the presence of clinical symptoms (**Figure 1A**). Stage 1 T1D includes patients with two or more diabetes associated auto-antibodies; stage 2 requires the presence of dysglycemia on top of islet autoimmunity; and stage 3 is considered as the classical T1D diagnosis; whereas stage 4 is long-standing disease (3). The presentation of T1D differs significantly between patients. The assumption that T1D is a typical children's disease proved to be wrong; the disease is diagnosed at any age at the same rate (4). Yet, children and adolescents present more often with full-blown ketoacidosis, whereas disease presentation in the adult population can be much more moderate, which could mislead to diagnosis of type 2 diabetes (T2D) (2). Serum c-peptide, a measure of endogenous insulin production, also widely varies between patients depending on the age and timeliness of diagnosis as an exponential drop is observed in the first 7 years after diagnosis, after which c-peptide levels remain stable over time (5). Diagnosis of T1D prompts the start of insulin therapy, which is injected by a pump or manually to manage blood glucose levels and, ultimately, for survival (2).

1.2. The burden of living with type 1 diabetes

T1D could pose a burden on patients, as managing glycemic control with insulin therapy is troublesome. Indeed, one in four adult patients feel a moderate-to-high emotional burden from diabetes (6), whereas in adolescents one in three are affected by diabetes-related distress (7). These studies conclude that diabetes-related stress could be associated with poor glycemic control as indicated by higher HbA1c (8). In turn, poor glycemic control could negatively impact academic achievements (9), whereas hypoglycemic episodes were associated with reduced verbal IQ in youth with T1D (10). This touches upon the conundrum of T1D care, namely that insulin is at the same time the best friend and foe of a T1D patient. Yet, even intensive glycemic control cannot always

prevent development of diabetic complications (11, 12). A better, safer, and stress-relieving therapy is needed that targets the cause of the disease instead of merely the symptoms.

1.3. Epidemiology

The sense of urgency for finding a cure for T1D has increased, since T1D incidence worldwide increased annually by 1.8% between 2002-2012 (13). Although T1D is historically known as a childhood disease, it can actually be diagnosed at any age (14). Still, an increased incidence is noted between the ages five and seven and at puberty (13, 15). In addition, incidence is higher in autumn and winter months and in countries with higher latitudes, such as Finland (16, 17). One common denominator of these risk factors is low sun exposure. Indeed, endogenous production of vitamin D3 is dependent upon ultraviolet B (UVB) radiation from the sun and a lack of vitamin D3 (VD3) and variations in the genes involved in the VD3 pathway have been associated with T1D development (18-20).

1.4. Genetics

Besides polymorphisms in the VD3 pathway, several other gene polymorphisms are associated with an increased risk of developing T1D (21). A common misconception regarding T1D, however, is that it is a heritable disorder that runs in families. In reality, T1D is a disease with polygenic predisposition and less than 10-20% of new cases have a family history of T1D (22, 23). Most of the genetic susceptibility is determined by the human leukocyte antigen (HLA) region on chromosome 6. HLA class II is expressed on antigen-presenting cells and functions as the carrier in which antigen is presented to T cells. Both susceptible HLA haplotypes (for instance DRB1*0401-DQB1*0302 and DRB1*0301-DQB1*0201) and protective HLA haplotypes (such as DRB1*1501-DQA1*0102-DQB1*0602) exist (24). The majority of other susceptibility genes are related to modulating the immune response (25). Therapies that could decrease the expression of these genetic risk markers, at least in some cell types, may be successful in treating or reducing the risk of developing T1D. Yet, a profound role for environmental and/or epigenetic factors in the development of T1D next to genetics should not be overlooked, as a study showed that there is 30-65% concordance between monozygotic twins after long term follow-up (26).

1.5. Epigenetics

Not solely are genes important, but also how they are regulated. Gene expression can be influenced by epigenetics. Epigenetics is a relatively new field which studies the heritable changes in gene expression that are not due to changes in the DNA sequence. Examples of epigenetic modifications are methylation of cytosines at CpG dinucleotides, histone

modifications and microRNAs that can all affect gene expression (27). It is not inconceivable that epigenetics could play a role in T1D, as T1D cannot fully be explained by genetics, and causative environmental factors are still elusive (28). Indeed, DNA methylation variability was increased in cord blood of newborns that would later develop T1D, compared to newborns that did not, suggesting that these epigenetic changes could contribute to T1D disease onset (29). In addition, epigenetic modifications were found in promotor regions of T1D risk genes in T1D patients compared to healthy controls (30, 31). Currently we are only scratching the surface of the implications of epigenetics on T1D disease onset and progression, as is exemplified by the paucity of literature on this subject. Besides, epigenetics could prove to be important in determining the stability of cellular therapies, as epigenetics has been implicated in establishing stable cellular phenotypes (32, 33).

1.6. Pathophysiology

T cells

Studies on the pathophysiology of T1D have historically focused on the immune system as the causative agent behind the destruction of β cells in the pancreas. Indeed, autoreactive CD8+ T cells are the most abundant immune cell type found in inflamed islets, followed by macrophages, CD4+ T cells, and B cells (**Figure 1A**) (34-37). Once CD4+ T cells are activated by presentation of antigen on HLA class II on antigen presenting cells, CD4+ T cells activate CD8+ T cells that kill insulin-producing β cells by recognizing islet antigens on HLA class I (35, 38, 39). Healthy individuals also have autoreactive T cells, but they are held in check by immune regulation by for instance T regulatory cells (Tregs) (40). The level of Tregs in T1D patients is similar to healthy individuals, but they are less capable of suppressing T cells, while effector autoreactive T-cells of T1D patients are more resistant to suppression, which may contribute to the progression of autoimmunity (41, 42).

The death of a β cell: revisiting the homicide / suicide model

At disease onset, 50-70% of islets are deprived of insulin staining, while inflammation is almost exclusively limited to insulin-containing islets, suggesting a targeted immune-mediated β cell attack (43, 44). According to the conventional model, islet autoreactive T cells target β cells and commit homicide of 'innocent' β cells, while an alternative model adds β cell suicide to the story (45, 46). This homicide/suicide model was first coined by Bottazzo in 1986, but since then many discoveries have shed a slightly different light on this scenario (47). It seems that β cells initiate interactions with T cells and T cells are merely acting on these requests, which would suggest more dialogue between the two parties rather than one-sided homicide or suicide. To illustrate this, β cells attract immune cells into the islet by secreting CXCL10 and expose themselves to T cells by

hyperexpressing HLA class I (**Figure 1A**) (48-50). Moreover, β cells present modified peptides which activate the immune system, as central tolerance in the thymus has not deleted T cells responsive to these "neo-antigens" (51). In a similar way, cancer cells express mutated antigens, which allows the immune system to remove the cancer (52). It is not yet clear what exactly triggers β cells to express these immune-activating neo-antigens. The prevailing hypothesis suggests a stress response of β cells, which induces the unfolded protein response and consequently post-translational modifications and defective ribosomal products (53-55). Proposed β cell stressors are cytokine-induced endoplasmic reticulum stress and hyperglycemia (56, 57).

In this sense, β cell death in T1D is not a case of homicide or suicide, but rather of T cell-assisted euthanasia of a stressed β cell calling for attention. Beta cell destruction is incomplete, however, as remaining insulin-positive β cells are found even in long-standing T1D (58). These β cells seem to be functionally impaired or hibernating, as they do not secrete insulin in response to hyperglycemia (59). This is an encouraging insight, as new therapies targeting β cell function may potentially wake up these hibernating β cells to secrete insulin again.

Stromal cells in the islet of Langerhans

The function of β cells could be supported by neighboring cells in the islet of Langerhans. Stromal cells, for instance, are embedded in the islets. Mesenchymal stromal cells (MSCs) are within the islets (**Figure 1A**) (60), whereas myofibroblasts surround the islets (61). In 1979 it was already known that fibroblasts promote the survival and function of β cells, although stromal cells have not received much attention up until recently (62). Besides the potential of MSCs to differentiate into β cells, MSCs improved the islet environment by secreting several growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) that could promote angiogenesis and β cell regeneration, respectively (63-65). In this regard, MSCs may be beneficial for β cell function, while at the same time they could contribute to maintaining immune balance in the islets (66). Thus, these on first sight innocuous cells may be used therapeutically in T1D to improve the islet environment.

Monocytes and dendritic cells

The destruction of β cells is set in motion by presentation of β cell-specific antigens to T cells by antigen presenting cells (APCs) (**Figure 1A**). Indeed, APCs are the true directors of the immune system orchestra. Conceivably, aberrant APC function may be implicated in the pathophysiology of T1D. Several cell types have antigen presenting capacities, but dendritic cells (DCs) are professional antigen-presenting cells, which could be derived from monocytes (67). Monocyte-derived DCs from T1D patients indeed showed

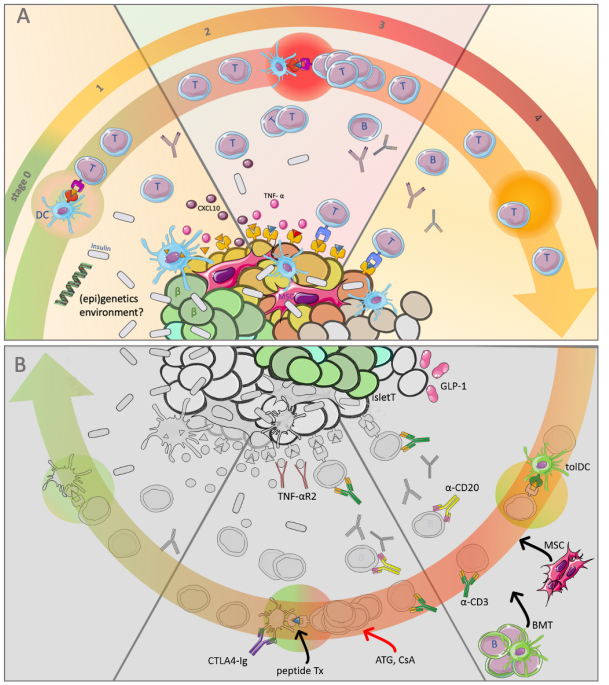

Figure 1: Natural History and Therapeutic Strategies in Type 1 Diabetes. (A) *The natural history and stages of Type 1 Diabetes. It is yet unclear which environmental triggers cause the onset of islet autoreactivity in genetically susceptible T1D patients. This onset is characterized by beta cell-antigen uptake and presentation by dendritic cells to autoreactive T cells. T cells then activate B cells to produce autoantibodies, which are detected in the blood. Once two autoantibodies are detected, a diagnosis of stage 1 T1D is prompted. Beta cells, in their turn, secrete the chemokine CXCL10 that attracts more immune cells into the islets. This causes more insulitis, which results in more dysfunctional beta cells and the initiation of dysglycemia and the start of stage 2 T1D. Consequently, cytokine production of infiltrating immune cells and antigen-specific cytotoxicity causes more beta cell death, which ultimately results in stage 3 T1D, necessitating exogenous insulin administration. In long-standing stage 4 T1D, beta cell mass is critically decreased, and what beta cells are still present are mostly in a dormant state not secreting insulin.* ***(B)*** *Therapies in T1D aim to reverse this vicious cycle of autoreactive T cell cytotoxicity and beta cell apoptosis by either targeting the immune system or the islets of Langerhans. In this animation, cellular, antigen-specific, and antibody therapies are depicted, next to drugs. CXCL10 is C-X-C motif chemokine ligand 10; ATG is anti-thymocyte globulin; CsA is cyclosporine A; peptide Tx is peptide therapy; MSC is mesenchymal stromal cell; BMT is bone marrow transplantation; tolDC is tolerogenic dendritic cell; GLP-1 is glucoagon-like peptide 1. Created in Biorender.com.*

differences compared to healthy subjects. Mainly decreased DC maturation and decreased capacity to stimulate autologous and allogeneic T cells was seen (68). Other studies corroborated that monocyte-derived DCs from T1D patients had abnormal NF-kB signaling and were less mature with low levels of activating molecules CD83, CD80, and CD86 (39, 68-70). These results seem counterintuitive as decreased DC maturation would impede activation of the immune system. Tolerance, however, is an active process, so these DCs with decreased maturation may still be able to activate T cells but not to regulate them. Besides functional differences, the frequencies of DCs differ, with higher levels of DCs at T1D diagnosis (39) and lower levels in new and recent-onset (71, 72) and established T1D, compared to healthy controls (73). Monocyte frequencies, however, were similar in T1D compared to healthy controls (72). In conclusion, both the function and frequencies of at least a subset of DCs have been claimed to be altered in T1D and modulating these cells may direct the immune system towards regulation.

B cells and antibodies

Although T cell-mediated β cell destruction is held to be the main cause of T1D, B cells and humoral autoimmunity should be considered as well. Several studies found that B cells infiltrate the islets in T1D (**Figure 1A**), which is even more prominent in patients diagnosed before the age of 7 (34, 74). Yet, a causal role for B cells and antibodies is still lacking (75). In fact, T1D was diagnosed in a patient with severe hereditary B-lymphocyte deficiency, illustrating that T1D can develop without the presence of B cells and antibodies (76). Nonetheless, β cell auto-antibodies have been found useful for diagnostic purposes and prediction of T1D development, even though 10% of T1D patients are negative (77, 78). If B cells do not cause T1D, why do they infiltrate the islets of T1D patients? One explanation could be that B cells are recruited secondarily by activated CD4 T cells and exacerbate T1D progression (34). Alternatively, B cells and the humoral response might regulate T cells in T1D, rather than contributing to β cell destruction. Several studies showed that islet auto-antibodies actually correlated inversely with T cell proliferation or activated CD8 T cell counts in T1D, corroborating this hypothesis (72, 79, 80). Furthermore, T cells secreted the inhibitory cytokine IL-10, but not the inflammatory cytokine IFN-γ, when recognizing an epitope that was shared with B cells (81). Thus far, however, no therapies have been successful in exploiting this postulated regulatory role of humoral immunity in T1D.

2. Therapies for type 1 diabetes

2.1. Rationale for curative type 1 diabetes therapies

After T1D diagnosis, insulin replacement therapy is started. Unfortunately, exogenous insulin is not a cure for T1D. Excessive amounts of insulin causes life-threatening hypoglycemia, whereas insufficient insulin subjects the patient to complications (82, 83). Retinopathy, neuropathy and nephropathy are long-term complications that are caused by periods of hyperglycemia. Although the incidence of these complications is reduced with intensive insulin treatment, there is no effective therapy today to prevent these (11, 12). Furthermore, meeting the HbA1c target of <7% remains a struggle for patients with 70% failing to achieve this and in a clinical trial this target was not even met despite strict intensive insulin therapy (83-85). Thus, mainstay insulin therapy does not satisfy the unmet need to improve glycemic control and decrease long-term complications in T1D patients. The rationale for curative T1D therapies shifts together with our understanding of the complexity and heterogeneity of the disease. Whereas the first T1D clinical trials primarily focused on suppressing the immune system, new strategies target multiple immune pathways, utilize antigen-specific strategies or cells as a vehicle and, finally, include β cells in the equation as well.

2.2. Immunotherapies for type 1 diabetes

Mono immunotherapies

The first immunotherapy trials assessed the effect of immune suppression by cyclosporine A that blocks T cell activity (**Figure 1B**). Two independent studies indeed showed that cyclosporine A reduced exogenous insulin needs for over 1 year. However, no lasting effect was obtained after cessation of therapy (86, 87), while cyclosporine A comes with the risk of nephro- and β cell-toxicity (88-90). Anti-CD3 antibodies such as teplizumab and otelixizumab also target the T cell (**Figure 1B**). Both antibodies improved c-peptide temporarily in a subgroup of patients with better baseline glycemic control, but not in the overall study population (91-93). Furthermore, in a preventative study, a two-week course of teplizumab was sufficient to delay the onset of T1D in high-risk individuals by two years (94). T-cell activation could also be blocked by preventing co-stimulation with the CTLA-4-Ig abatacept (**Figure 1B**). Abatacept delayed c-peptide decline in recent-onset T1D by approximately 10 months, but sustained treatment could not prevent subsequent loss in c-peptide. The authors concluded that T cell activation might be less prominent over time, as six months after start of abatacept the rate of decline was similar in the treatment group as control (95). Similarly, rituximab, an anti-CD20 antibody targeting B cells (**Figure 1B**), delayed c-peptide decline in a small subset of patients but was unable to result in sustained remission (96, 97). Treatment with alefacept, a drug that inhibits activated T-cells, resulted in sustained preservation of c-peptide secretion up to 15 months after

cessation of therapy (98, 99). Other therapies, such as the TNF-α inhibitor etanercept and Bacillus Calmette-Guerin (BCG) vaccination, have shown improvements in c-peptide levels at least in some subjects (100, 101), whereas anakinra, an IL-1 receptor agonist, and intravenous immunoglobulin (IVIG) did not (102, 103).

Together, these trials emphasize the notion of heterogeneity between T1D patients in terms of response to treatment, as only subgroups of patients responded to many of these targeted mono therapies. Nonetheless, all patients could conceivably be subject to side effects posed by these drugs, as most of them cause nonspecific immune suppression. The abatacept trial illustrated that the optimal time to interfere might be earlier in the disease process and this could be dependent upon the intervention used. Thus, it is crucial to identify the right patient population that would benefit from the treatment as well as the right timing and length of intervention for each drug regimen separately. A way to possibly circumvent these problems is to target several pathways at once, so that more patients will experience efficacy for a longer period.

Combination immunotherapies

After the somewhat disheartening results from monotherapy trials, a change of tack was needed. The facts were obvious: T1D is a complex, multi-system disease that is heterogenous between patients. The belief to cure or counter this disease with a monotherapy in all patients was perhaps wishful thinking. Nonetheless, subgroup effectivity of monotherapies should not be disregarded, but combining therapies that target multiple pathways may broaden the scope of effectivity to more patients and may empirically reduce dosing and side effects (104).

Unfortunately, the first combination trials were unsuccessful and even resulted in increased c-peptide decline in the case of rapamycin and interleukin-2 (IL-2) or adverse events in the case of mycophenolate mofetil (MMF) with daclizumab (DZB) (105, 106). Although low-dose anti-thymocyte globulin (ATG) reduced c-peptide decline and improved HbA1c, the combination of ATG with granulocyte colony-stimulating factor (GCSF) did not reduce c-peptide decline compared to placebo after 2-year follow-up (107, 108). A more drastic approach relied on a modified autologous hematopoietic stem cell transplantation using GCSF and cyclophosphamide to mobilize cells and cyclophosphamide and ATG to ablate the immune system (**Figure 1B**). This method had the unprecedented result of achieving insulin independence in the majority of patients after more than 2 years follow-up with even longer lasting insulin independence in a subgroup with low autoimmunity at baseline (109, 110).

Theoretically, combination therapies seem sensible in the context of T1D, but there is much to learn. These trials emphasize, once again, that the timing, patient population,

and the specific combination of therapies matter. What the magical combination of therapies would be is still unclear, but combining antigen nonspecific drugs that attack a similar pathway warrants increased side effects. Indeed, the future might be in combining immunomodulatory drugs with antigen-specific drugs.

Antigen-specific immunotherapies

Antigen-specific immunotherapies could be one of the most promising strategies to treat T1D, as this disease is characterized by a very specific attack on β cells by an autoimmune insult targeted at their autoantigens (**Figure 1B**) (111). In general, antigen-specific therapies aim to induce an immune response to specific antigens, instead of suppressing immunity as a whole and in the latter case, risking infections and impaired cancer surveillance. In immune activating therapies, the antigen is conventionally given with an adjuvant, which could either be a cell (discussed in the next paragraph) or another type of immune activator or engager (112). Adjuvant optimization is key to the success of any antigen-specific therapy and could determine whether the therapy is immune activating or inducing tolerance to the antigen, as is desired in T1D. Trials with oral insulin in this regard showed beneficial immune modulation in a subset of at-risk individuals, although no overall effect was seen (113-115). Dosing and the choice of antigen could be improved (111). Indeed, c-peptide levels were maintained after therapy with the more immunogenic proinsulin peptide and an IL-10-driven antigen-specific response was noted (116). Other antigen-specific therapies were also found to be safe and conferred beneficial effects to at least a subgroup of patients (117-120). A new avenue was opened when antigen-specific therapies were combined with immunomodulatory therapies. For example, the combination of intralymphatic glutamic acid decarboxylase (GAD)-alum and vitamin D showed promising results with a decrease in HbA1c and maintained c-peptide levels in a small pilot study, but it lacked a control group (121). Several other trials are now being conducted with different drug additions to GAD-alum, such as etanercept and GABA (clinicaltrials.gov; NCT02002130; NCT02464033). Finally, the risk of inadvertent immune activation with antigen therapy should be acknowledged and this risk, together with efficacy, could be improved with adjuvant optimization by, for instance, optimizing cellular therapies that could carry the antigen.

Cellular immunotherapies

Cellular therapies have the promise of reinstating equilibrium in a more natural way than a specific targeted drug, as cells have a broad array of functions and feedback mechanisms. Indeed, cells secrete multiple factors instead of just modulating one factor by for instance blocking it with a monoclonal antibody. Often cells and their functions are plastic, which accounts for their strength as they adjust to their environment, but it comes with a caveat of the possibility of an ‘‘unstable’’ drug (122). In general, cellular therapies

can either consist of unaltered cells to repopulate a cell population that was found decreased in a disease or of cells that are altered in a way to make them more fit to combat the disease. The added advantage of using autologous cells is that there is no risk of rejection (123). Examples of the latter category are T regulatory cells (124, 125), tolerogenic dendritic cells and activated mesenchymal stromal cells.

Tolerogenic dendritic cells

Dendritic cells (DCs) are crucial to directing an adaptive immune response. Their antigen presenting capacity is mostly known to induce a pro-inflammatory immune response against non-self-antigens. In the thymus, however, DCs can also induce tolerance against self-antigens. Autoimmune disease in this respect seems to be due – at least in part – to DC mediated self-antigen presentation in an immune activating setting (126). As mentioned previously, dendritic cells of T1D patients indeed had an abnormal activation status, compared to healthy individuals (69, 71, 127). Thus, converting autologous DCs into tolerogenic cells (tolDCs) would be an attractive way to engage the immune system with a peptide therapy (**Figure 1B**). The first phase I clinical trial with autologous tolDCs made ex vivo was deemed safe, although this was without peptide added (128). TolDCs can be produced by multiple methods, including pharmacologically by for instance dexamethasone and VD3 treatment, or by increasing immunomodulatory molecules such as IL-10 or downregulating co-stimulatory molecules via gene therapy (129). VD3 is particularly poised to reinstate the balance in the immune system, as it is a known immune modulator and found to be deficient in T1D patients (130-132). Furthermore, VD3 is advantageous as it has been used as a dietary supplement for decades and safety was secured in T1D trials, which concluded that VD3 supplementation in early childhood may reduce the risk of developing T1D later in life (133, 134). VD3 has synergistic effects with dexamethasone, which is widely used in the clinic as an immunosuppressant and blocks DC maturation (135). An area of concern of pharmacologically induced tolDCs is their stability, however, as tolDCs could potentially convert to a pro-inflammatory phenotype and this should be addressed to safeguard its translation into the clinic. Furthermore, it should be validated that autologous tolDCs from T1D patients are similar to tolDCs from healthy individuals.

Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are of interest as they are believed to be inherently immunomodulatory (**Figure 1B**) (136). Furthermore, the fact that MSCs are already used in the clinic could expedite its translation for T1D treatment (137). MSCs secrete immunosuppressive factors such as indoleamine 2, 3-dioxygenase (IDO) and express immune inhibitory factors such as PD-L1 (138, 139). Upon activation with pro-inflammatory cytokines the immunosuppressive properties of MSCs are thought to be

enhanced (140). There is a fear, however, that this manipulation (with pro-inflammatory cytokines) could result in inadvertent activation of the immune system, as was similarly feared for tolDC therapy (141). Besides this, MSC therapy is not antigen-specific. In conclusion, it is important to investigate the effect of pro-inflammatory cytokines on MSCs' immunosuppressive phenotype and examine the potential of MSCs to become antigen-specific.

2.3. Beta cell therapies

As argued before, immunotherapy may not suffice to cure T1D, as β cells appear actively involved in their own demise. The realm of β cell therapies has mainly consisted of efforts towards β cell replacement and to a lesser extent toward β cell recovery.

Beta cell replacement

The first attempt to replace β cells in T1D patients was successfully achieved by the advent of islet transplantation in the 1980's (**Figure 1B**) (142). Although this remains an important therapy for rare patients suffering from hypo-unawareness and uncontrolled blood glucose levels, the scarcity of islet donors and the immune suppression needed to prevent graft rejection halt its wide application in T1D (143). In addition, the viability and successful engraftment of islets are of concern and often times multiple islet infusions are needed to achieve insulin independence (144-147). In this sense, β cell recovery strategies could in addition be used to improve islet transplant viability and function. MSCs are a good example of this, as they improved β cell function in T1D patients by themselves and could be used in combination with islet transplantation as well (148, 149). Other strategies to replace β cells consist of producing β cells from other types of cells, such as stem cells, and are reviewed elsewhere (142).

Beta cell recovery

The field of β cell recovery therapies is still in its infancy. Although extrapolation from T2D therapies should be possible, currently there are no FDA-approved drugs for T1D therapy that specifically target the β cell. In fact, a systematic review of T1D clinical trials identified 2090 registered trials in 2018, of which 212 were investigational drugs and only 30% of these 212 trials focused mechanistically on the β cell (150). This suggests that there is a sea of opportunity for innovations regarding β cell recovery and survival. The glucagon-like peptide 1 (GLP-1) signaling pathway is by far most researched with 72% of clinical trials in β cell recovery dedicated to it (150). Most drugs targeting the GLP-1 pathway are analogues of GLP-1, such as liraglutide, and have been used in T2D management for more than a decade. In T1D, liraglutide has shown promising clinical results as well (**Figure 1B**) (151-154). Mechanistically, GLP-1 analogues could work by promoting β cell proliferation (155, 156) and glucose stimulated insulin secretion by the β cell (157).

3. Aims and outline of thesis

Drawing from the analysis of recent and new immune modulating and β cell therapies, my thesis aims to decipher promising treatment paradigms for T1D. Chapter two and three describe two studies in which T1D was successfully reversed. The first study involves a drastic reset of the immune system by autologous hematopoietic stem cell transplantation, whereas the second study is a case report of successful reversal of T1D in the setting of IVIG treatment. As these treatment strategies are associated with morbidity or only incidental success, respectively, other therapies that aim to reinstate a subtler immune balance are discussed in chapter four and five. Therein, the possibility of using tolerogenic dendritic cells or activated mesenchymal stromal cells as antigen-specific immunomodulation in T1D is discussed. Chapter six engages the islets of Langerhans as targets for therapy. In this chapter, MSCs show additional beneficial potency to improve the islet microenvironment. Chapter seven summarizes these different strategies and puts these in perspective, while their significance to future T1D therapies is discussed.

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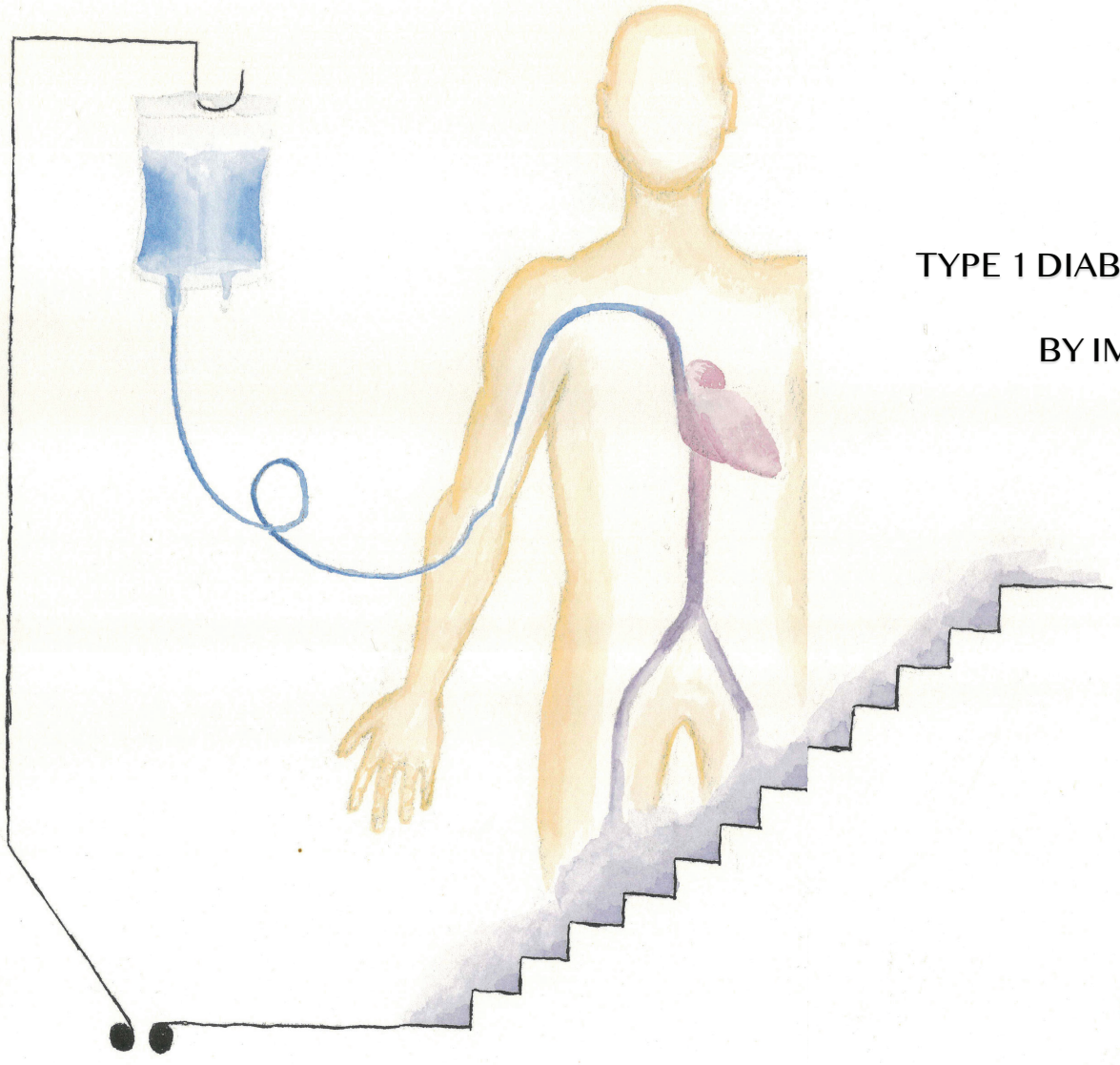
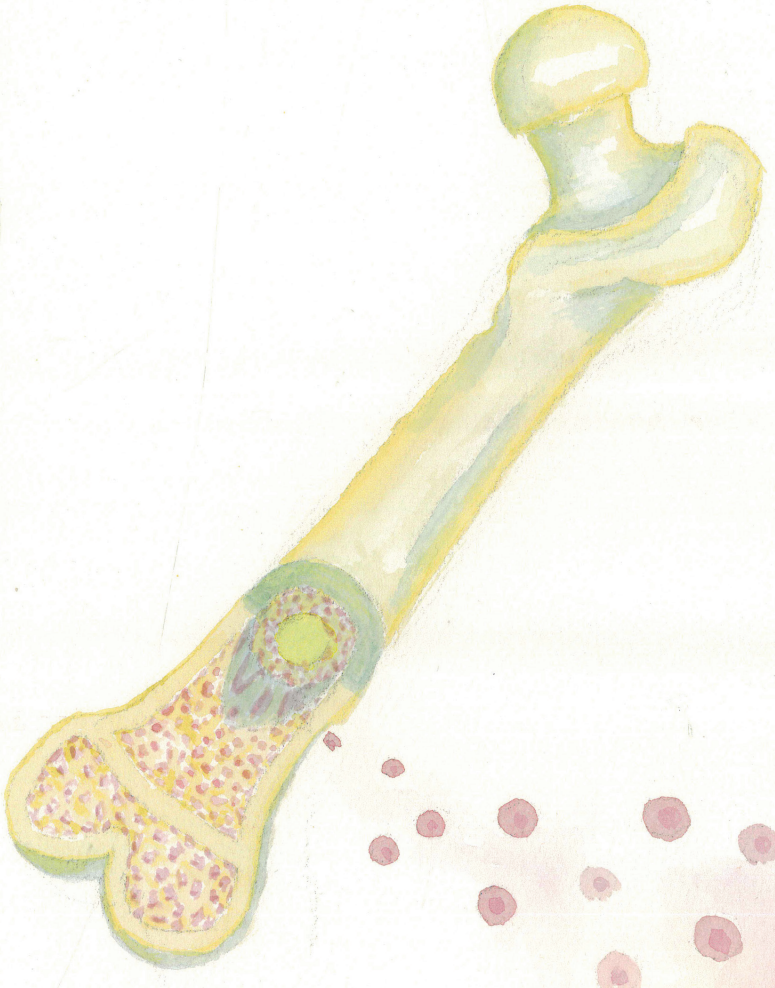


PART I

TYPE 1 DIABETES CAN BE REVERSED

BY IMMUNOTHERAPY



Chapter 2

A future for autologous hematopoeitic stem cell transplantation in type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by destruction of insulin producing β-cells in the pancreas. Standard of care therapy consists of life long symptomatic insulin treatment and in rare and severe cases patients undergo islet transplantation (1). Until today, autologous hematopoietic stem cell transplantation (aHSCT) proved to be the only intervention therapy for T1D reaching complete and sometimes even lasting remission (2–7). In spite of many other immunotherapies assessed around the globe, none matched the clinical efficacy of aHSCT (8, 9). Indeed, aHSCT had insulin-independency as primary end-point, rather than delayed loss of insulin production or decreased insulin needs. aHSCT is already widely and successfully used as a treatment for hematological malignancies (10, 11). Interestingly, one diabetic patient, when treated with aHSCT for multiple myeloma, became insulin independent (12). aHSCT was evaluated as a treatment for several autoimmune disorders as well, such as rheumatoid arthritis (13), systemic sclerosis (14, 15), multiple sclerosis (16), and juvenile idiopathic arthritis (17). By 2012, up to 3,000 aHSCT had been performed for autoimmune diseases (18). Yet, in the case of T1D, aHSCT remains controversial (19–21).

Indeed, the use of aHSCT as a strategy to cure T1D has been received with mixed enthusiasm. Concerns were raised about the short follow-up, the possibility that a positive effect of aHSCT may be attributable to a honeymoon phase and the absence of a placebo-treated trial arm for comparison (19, 21). Furthermore, the ethics of including minors in the trial was being questioned (19). Although valid at the time, these concerns have all since been addressed, as will become evident in the following paragraphs.

aHSCT in T1D

The rationale behind using aHSCT in autoimmune diseases is to halt autoimmune destruction of the targeted tissue and reestablish tolerance. While the mechanism by which this is achieved remains incompletely resolved, the importance of a diverse T-cell receptor repertoire (22), thymus reactivation (23), and the number of regulatory T-cells (Treg) has been established (24).

The first evidence to demonstrate that aHSCT can reestablish tolerance in new-onset T1D patients comes from Voltarelli et al. (25, 26). Recent-onset (<6 weeks) T1D patients were included to undergo aHSCT with mobilized [cyclophosphamide (2.0 g/m2) and granulocyte colony-stimulating factor (10 μg/kg/day)] peripheral blood-derived hematopoietic stem cells after an intermediate-intensity conditioning regimen consisting of cyclophosphamide (200 mg/kg total) and rabbit antithymocyte globulin (4.5 mg/kg total). Similar mobilization and conditioning regimes were used in other discussed studies, unless mentioned otherwise. In total, 25 patients were included, of which 21 were treated according to protocol and became insulin independent, for a median of 43 months (2); a

result unmatched by any intervention therapy up until this point. These results were substantiated independently around the world, accomplishing insulin independence in all studies, with maximum insulin independence ranging from 38 to 56 months and increasing with further follow-up (3-7). These studies prove that aHSCT is a promising therapy for T1D, while providing crucial and unique metabolic and immunological data of T1D patients in remission (27, 28).

Balancing the risk of aHSCT with the risk of diabetes-associated complications

Depending on the intensity of the conditioning regime, aHSCT can cause a wide range of complications. In the T1D trials (2–7), these ranged from relatively mild symptoms such as febrile neutropenia, nausea, and alopecia to more severe complications such as de novo autoimmunity and systemic infections, which in one case resulted in an unfortunate death (7). Temporal oligospermia was witnessed in some of the studies, but not all. Of note, multiple children have been conceived after aHSCT. Apart from these complications, there is also a concern of increased risk of malignancies after aHSCT, particularly myelodysplasia. With allotransplantation, this risk is well established and can be attributed to the heavy conditioning regime, while this regime is much milder in the autologous setting for autoimmune diseases. Furthermore, in contrast to aHSCT as a treatment for malignancies, stem cells of T1D patients have not sustained any damage from previous chemotherapy. Consequently, the incidence of malignancies was reported to be lower, although further prospective studies with longer follow-up and proper control groups are warranted to assess if these malignancies are aHSCT related (29).

Containment of adverse events from aHSCT is constantly improving as illustrated by decreased morbidity and mortality to <1% (30). Furthermore, in the setting of T1D, it will be performed in relatively young and otherwise fit subsets of patients with a low to intermediate conditioning regimen (2, 31), associated with reduced risk (29) without compromising treatment efficacy. This was attested by a recent trial exploring the possibility of a simplified method of aHSCT in an outpatient setting, with a conditioning regime consisting of cyclophosphamide (2.0 g/m2 total) and fludarabine (120 mg/m2 total), still reaching 44% prolonged insulin independence for up to 56 months and beyond, without significant adverse effects (4).

To make a compelling and fair case of aHSCT in T1D, the complications of aHSCT need to be juxtaposed with the short- and long-term complications of T1D. It is important to realize that acute and possibly life-threatening events related to T1D and insulin treatment such as a hypoglycemic coma (32) and diabetic ketoacidosis (DKA) (33) are not uncommon. Indeed, T1D remains a deadly disease, where insulin therapy merely provides palliative care. In addition to a significantly reduced life expectancy, T1D also imposes

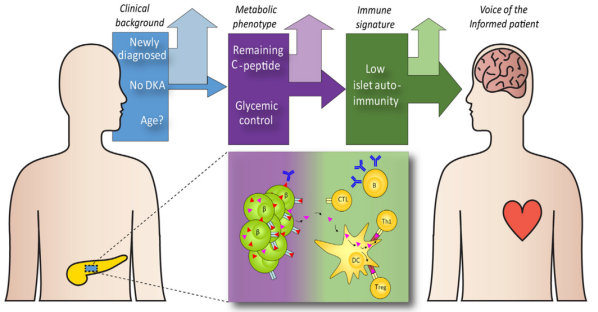

Figure 1: Guidance on the selection of type 1 diabetes (T1D) patients for autologous hematopoietic stem cell transplantation (aHSCT). *aHSCT is unlikely to benefit all T1D patients. Factors that may help selecting the preferred candidates include the clinical background [disease duration, age, and diabetic complications, such as diabetic ketoacidosis (DKA)], metabolic features [remaining functional beta-cell mass (ß), glycemic control, HbA1c] and immunopathogenic features [the number and type of islet autoantibodies, the frequency and specificity of islet-autoreactive cytotoxic T lymphocytes (CTL), and other effector (Th1) and regulatory (Treg) immune cells, and cytokine profiles]. With the opportunity to identify patient subgroups with particularly great or smaller chances for clinical benefit, we propose that we engage the patient community to guide shared decision-making.*

severe and often lifelong negative impact on the quality of life of T1D patients. The major burden of the disease is caused by long-term micro- and macrovascular complications, with T1D still being a main cause of end stage renal disease and non-inherited blindness (34, 35). Even with optimal education and state-of-the-art treatment options, good glycemic control is not achieved in the vast majority of patients (36). This is of particular importance, since good glycemic control early in the course of the disease reduces long-term complications and preserves endogenous insulin production (37). Interestingly, patients that experienced a honeymoon phase showed significantly less macrovascular complications after 7 years of follow-up (38, 39). This could imply that a similar effect can be expected from an aHSCT induced prolonged period of insulin independence.

Importantly, side effects are inherent to immunotherapy. The adverse events of, for instance, DMARD, TNF blockers, sirolimus, cyclosporine, azathioprine, prednisone, thymoglobulin, alemtuzumab, or imatinib, all considered in the context of T1D, are certainly not negligible.

Clinical outcome of aHSCT corresponds with the degree of islet autoreactivity before therapy

Currently, after almost 15 years of experience in the application of aHSCT for the treatment of T1D, much knowledge has been gained about the mechanism of action of aHSCT and, concomitantly, about which patient population benefits most (2, 3, 5–7, 27, 28, 40–42).

Earlier this year, the first aHSCT in T1D trial reported its ad hoc analysis with a mean follow-up of 67.5 months (some patients remain insulin-independent beyond 106 months) and included 25 patients (2). HLA-A2 positive patients were divided into low and high cytotoxic T lymphocytes (CTL) autoreactivity groups according to the cumulative frequencies of islet-specific CTLs at baseline. Low CTL autoreactivity associated with higher c-peptide levels after aHSCT compared with high CTL autoreactivity. Furthermore, while 83% of patients in the high CTL group had resumed insulin therapy at 24 months after aHSCT, all patients with low frequencies of islet-autoreactive CTLs at baseline remained insulin independent. In addition, patients were divided into those with "short-remission" and "prolonged remission" depending on whether they were insulin-free for less or more than 3.5 years after aHSCT, respectively. A trend was seen of persistently lower cumulative frequencies of islet-specific CTLs in the prolonged remission group compared with the short-remission group. This outcome may point that the conditioning regimen with thymoglobulin was insufficient to deplete auto-reactive T-cells. Diabetes relapse could then result from clonal expansion of autoreactive CTLs that escaped the conditioning procedure. In any case, these immunological parameters associated with superior or inferior clinical outcome of aHSCT before therapy point to patient and disease heterogeneity and present a good case for personalized and precision medicine in which tailoring the conditioning therapy might lead to more effective reversal of islet autoimmunity.

Additional evidence in favor of an immunogenic heterogeneity that relates to the outcome of aHSCT came from a study of 13 patients that was conducted in China with a mean follow-up of 42 months (5). Expressing more than one preexisting autoantibody negatively correlated with the preservation of beta-cell function as quantified by c-peptide levels. Yet, a larger study including 123 patients with a mean follow-up of 16 months found no difference in baseline presence of any of the autoantibodies between responding and non-responding patients (27). Serum levels of interleukin-10, interleukin-4, transforming growth factor-β, and fasting c-peptide after aHSCT correlated with the number of infused CD34+ cells, whereas tumor-necrosis factor-α (TNF-α) and insulin doses showed an inverse relation. Furthermore, prolonged insulin-free survival was

negatively correlated with baseline TNF-α levels, which may provide another suitable negative predictor of prolonged remission (3).

In summary, current clinical evidence points to heterogeneity between patients and in disease, as well as provides immune correlates of disease remission or relapse that may offer opportunity for patient selection, precision medicine, and guidance for tailored immunotherapy following aHSCT.

The success of aHSCT in relation to preexisting functional beta cell mass

Besides a baseline immune signature, *post hoc* analyses have revealed the importance of preexisting beta-cell mass for the outcome of aHSCT (27). One small study (5) found that the baseline c-peptide level was a positive predictor of post-aHSCT c-peptide levels, which was corroborated by other, larger studies (3). The largest study including 123 patients stratified subjects into a responder group and a non-responder group according to the presence of a post-aHSCT clinical response assessed by a β-score (27). The β-score is mainly used in the islet transplantation setting and consists of four components: fasting plasma glucose, HbA1c, c-peptide, daily insulin use or usage of oral hypoglycemic agents. The β-score was already significantly higher at baseline in responders compared with non-responders. Moreover, baseline fasting c-peptide levels proved to be an effective positive predictor of prolonged remission and the age of onset of diabetes a negative predictor. Obviously, baseline c-peptide levels are an indication of functional β-cell mass (27), although increasing evidence points to a disconnect between beta-cell mass and function in the case of diabetes (43, 44). β-Cell regeneration may occur until adolescence, after which regenerative capacity appears to stagnate (45). Indeed, early intervention within 6 weeks after diagnosis of T1D led to remission in the vast majority of cases, whereas later intervention achieved remission in less than half of the cases (42), suggesting that timely therapy matters.

The influence of DKA before aHSCT on clinical outcome could be substantial (6). Indeed, DKA at diagnosis has been associated with lower c-peptide levels, higher insulin needs and HbA1c levels, suggesting lower remaining β-cell function (46). Yet, another trial including 24 patients with 52 months as a mean follow-up found no relation between duration of insulin independence and the time from diagnosis to AHSCT, baseline c-peptide levels, nor number of CD34+ cells (7).

To summarize, patients with sufficient beta-cell function at baseline, no DKA at diagnosis, and treated early after diagnosis appear to benefit most. These characteristics all point toward the pivotal role of remaining functional beta-cell mass for success of aHSCT in T1D (27). To verify whether the age of onset matters (3), inclusion of minors in trials of aHSCT in T1D would be required. The potential capacity to regenerate their beta cells would

further support considering young patients to offer this intervention therapy. Teenagers are a particularly challenging population to treat as diabetes-related distress, which is present in one-third of adolescents with T1D, is linked to poor glycemic control (47–49). Consequently, 84% of teens do not reach target HbA1c levels (36), which jeopardizes their future health with regards to increased long-term complications, but also their career perspectives (50).

Selecting eligible patients for aHSCT in T1D

Understanding which patient groups respond better to aHSCT and why, enables us to transform aHSCT from a general therapy to personalized medicine, thus envisioning a future of aHSCT in T1D. Yet, we contend that the choice for aHSCT as therapeutic option is not confined to the care providers. The voice of the patient is equally relevant, both in terms of refusing the risk for treatment related adverse events or accepting these in favor of temporal disease remission, preservation of beta-cell function, and reduced risk of diabetic complications. In case of minors, parents face the difficult task of weighing the best therapy for the patient in consultation with the care provider, which makes careful information provision even more important. We envision a future in which care providers, in dialog with the patient and caregivers, use a framework of evidence-based risk assessment to assess whether aHSCT is a viable option (see **Figure 1**).

Conclusion

While aHSCT will not be the magic bullet universally curing T1D, there is a promising future for its implementation in a distinct group of patients (20). Indeed, none of the alternative intervention strategies match, or even get close to, the clinical outcome achieved in a considerable number of patients treated with aHSCT. We propose that this patient group should be identified, diligently informed and offered the possible benefits of an extended period of insulin-free and burden-free survival, while medical science continues their pursuit of developing alternative intervention strategies for those less eligible, or declining, aHSCT. T1D enters the era of personalized medicine.

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Chapter 3

Relapsing / remitting type 1 diabetes

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Abstract

Aims/hypothesis

Type 1 diabetes is believed to be an autoimmune disease associated with irreversible loss of insulin secretory function that follows a chronic progressive course. However, it has been speculated that relapsing/remitting disease progression may occur in type 1 diabetes.

Methods

We report the case of an 18-year-old girl with Graves' disease, chronic inflammatory demyelinating polyneuropathy (CIDP) and multiple islet autoantibodies, presenting with relapsing/remitting hyperglycaemia. Peripheral blood mononuclear cells were analysed for islet autoimmunity.

Results

There were two instances of hyperglycaemia relapse during CIDP flare-ups that required insulin therapy and remitted after i.v. immunoglobulin (IVIG) therapy improving neurological symptoms. A diagnosis of type 1 diabetes was assigned on the basis of insulin need, HbA_{1c} and islet autoantibodies. Insulin requirements disappeared following IVIG treatment and peaked during CIDP flare-ups. Pro- and anti-inflammatory cytokine responses were noted against islet autoantigens.

Conclusions/interpretation

We provide clinical evidence of relapsing/remitting type 1 diabetes associated with IVIG treatment and the regulation of islet autoimmunity. Despite sufficient residual beta cell mass, individuals can experience episodes of impaired glycaemia control. This disconnect between beta cell mass and function highlighted by our case may have implications for the use of beta cell function as the primary endpoint for immune intervention trials aiming to protect beta cell mass rather than function. Immune modulation may restore beta cell function and glycaemic control

Introduction

Studies on cohorts of people with recent onset type 1 diabetes suggest steadily declining pancreatic beta cell function over time. However, these data may not necessarily reflect the dynamics of disease progression in individuals (1, 2). Here we present an individual with relapsing/remitting type 1 diabetes with regulated islet autoimmunity.

Methods

In February 2013, a 15-year-old girl presented with muscle weakness, numbness in the arms and legs, and areflexia. Blood tests revealed elevated blood sedimentation rate, thrombocytosis and initially normal serum glucose. Elevated protein levels were observed in the spinal fluid. Collectively indicating a diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP). She was treated with i.v. immunoglobulin's (IVIG) for 5 days with good results. CIDP is characterised as a relapsing disorder which develops over a period of several weeks. The classical clinical pattern of the disease is described by the American Academy of Neurology and consists of involvement of proximal and distal limbs and of both motor and sensory fibres (3).

Proliferating peripheral blood mononuclear cells (PBMCs) were measured by ^{3}H-labelled thymidine uptake using a previously published method (4). PBMCs were pulsed with antigens relevant to type 1 diabetes (preproinsulin [PPI], islet antigen-2 [IA-2] or glutamic acid decarboxylase 65 [GAD65]). Tetanus toxoid was used as a positive control. Human serum albumin was used as a negative control, to which the proliferation data were normalised by subtracting the negative control values from the diabetes-relevant antigen values. Recombinant PPI, IA-2 and GAD65 were purified from *E. coli* and tested negatively for endotoxin contamination. Supernatant fractions from proliferation cultures were collected and cytokine production measured using a nine-plex Luminex kit (Biorad, Veenendaal, the Netherlands). Autoantibody titres were measured as previously described (4). Informed consent was given by the individual.

Results

In September 2014 (18 months later) the girl experienced a CIDP relapse. She displayed similar symptoms as previously described. She was re-admitted to hospital and received a second round of IVIG treatment (2 g/kg over 5 days). This time serum glucose was elevated to 24 mmol/l (432 mg/dl), HbA_{1c} was 7.6% (60 mmol/mol) and fasting C-peptide was normal (0.57 nmol/l). Furthermore, she tested positive for islet-specific (GAD, IA-2 and zinc transporter 8 [ZnT8]) as well as thyroid-specific (thyroid peroxidase and thyrotropin receptor) autoantibodies, and ketone bodies were found in urine, prompting the diagnosis of Graves' disease and type 1 diabetes following the criteria defined by the ADA. Her HLA type is also associated with an increased risk for type 1 diabetes and Graves' disease (HLA-DR3,11; -DQ2,7). She was started on daily insulin injections that normalised her blood glucose levels. However, within 1 month of IVIG treatment her basal insulin needs dropped. Five months later her insulin needs disappeared altogether, her HbA_{1c} normalised and her serum glucose levels remained within the normal range with no further treatment or dietary restrictions (**Figure 1**). Her thyroiditis did not respond to IVIG.

In August 2015, 5 months after the last insulin administration, she was re-admitted to hospital for a third CIDP relapse (**Figure 1**). Serum glucose levels were elevated at the time of hospitalisation. Insulin treatment was re-established, and she underwent another round of IVIG therapy. Again, her basal insulin needs dropped following completion of IVIG treatment and insulin administration was stopped completely 1 month after hospitalisation.

A blood sample that was collected within 1 month of her most recent round of IVIG therapy was analysed for islet autoantigen responses and the presence of T regulatory cells (Tregs) (**Figure 1**). T cell proliferation was only noted in response to IA-2, but cytokine responses were detectable against both IA-2 and PPI. Strikingly, proinflammatory cytokine responses (IFN-γ and IL-17) were matched by anti-inflammatory IL-10 production (**Figure 1**). Normal levels of Tregs were found (9.2% $CD25^{high}$ $CD127^{low}$ $FOXP3^{+}$ Tregs). Serum analysis revealed circulating autoantibodies to GAD65 (221 U/ml, range < 4 U/ ml), IA-2 (1.34 U/ml, range ≤ 1 U/ml) and ZnT8 (287.5 U/ml, range < 15 U/ml) autoantibodies. Islet cell antibody and insulin autoantibody levels were negative. At the time of this report (February 2017), the individual was normoglycaemic, without insulin need and with stable thyroid function.

Conclusions

Type 1 diabetes is classically described as a progressive disease with irreversible loss of insulin secretory function due to the destruction of beta cells. The present case provides evidence that the natural history of the loss of beta cell function may be more dynamic than the traditional model assumes. We describe relapsing and remitting clinical symptoms in an individual with type 1 diabetes. The capacity to regain complete metabolic control over the course of 18 months implies that residual beta cell mass is sufficient to restore euglycaemia.

It remains unclear to what extent the loss of beta cell function resulted from the loss of beta cell mass as insulin production was completely restored. The current case teaches that insulin insufficiency is not a direct measure of beta cell destruction or mass. Consequently, insulin need as a measure of impaired beta cell function may underestimate beta cell mass.

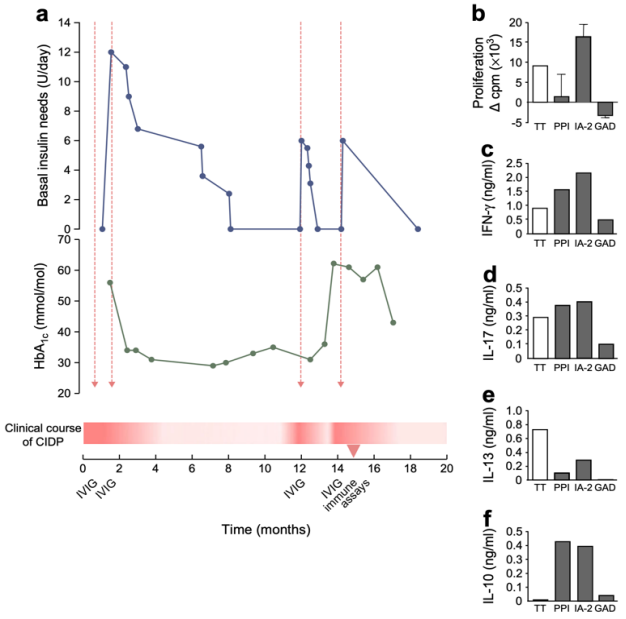

Figure 1: Time course of CIDP disease progression, immunotherapy and type 1 diabetes as defined by insulin need and HbA_{1c} (A) *During the patient's most recent flare-up of CIDP, a blood sample was drawn and analysed for T-cell reactivity to the islet autoantigens PPI, IA-2 and GAD65 (GAD), as well as T cell responses to tetanus toxoid (TT) as a control for recall immunity to a vaccine antigen unrelated to type 1 diabetes* ***(B-F)****. Proliferative responses to PPI were suppressed despite pro-inflammatory (IFN-γ, IL-17) and anti-inflammatory cytokine production in response to this islet antigen. T cells responded both to IA-2 by proliferation and cytokines, whereas no T cell responses were detectable against GAD65 despite the presence of serum autoantibodies against this protein (not shown). Dashed red arrows indicate IVIG administration. The timeline starts on September 2014 (t = 0 months) and ends on May 2016 (t = 20 months). The clinical course of CIDP is depicted as a red graded fill, in which the more intensely red areas are flare-ups and white areas are periods of remission. To convert values for HbA_{1c} in mmol/mol into % units, multiply by 0.0915 and add 2.15. Proliferation was normalised to the response in control wells with culture medium and human serum, i.e. without a diabetes-associated antigen (9.5 ± 1.0 [SD] cpm × 10^3). Cytokine production in control wells was as follows (in ng/ml): IFN-γ, 0.059; IL-17, 0.003; IL-13, 0.006; IL-10, 0.026*

Suppressed T cell proliferation and robust IL-10 responses to islet autoantigen point to a capacity of the immune system to counter loss of immune tolerance to islets. These residual immunological tolerance mechanisms may be boosted by immunotherapy, even after clinical diagnosis of type 1 diabetes. We previously demonstrated that a T cell response to islets is dominated by IL-10 in non-diabetic donors with a high genetic risk of developing type 1 diabetes, whereas individuals with type 1 diabetes who respond to islet antigens by producing IL-10 develop their disease later in life than those lacking IL-10 responses (5). Intriguingly, no T cell responses were detectable to GAD65 despite the presence of autoantibodies to GAD65, underscoring a previously noted inverse correlation between T and B cell responses to islet autoantigens (6).

While IVIG might affect peripheral insulin resistance by reducing inflammation, we favour the possibility that immunoregulation contributed to the relapsing and remitting course of diabetes in this individual. Indeed, T cell proliferation to PPI was low and accompanied by IL-10 production, while the immune response against IA-2 was also accompanied by IL-10 production. IVIG may have contributed to immune regulation of diabetes reversing dysfunction of otherwise viable beta cells and restoring normoglycaemia in addition to affecting CIDP. Unlike type 1 diabetes, pathogenic autoantibodies have been identified in CIDP, which could be neutralised by IVIG (3). Comorbidity of CIDP and type 1 diabetes has been reported previously (7). Yet in contrast to our present case, all previously reported instances indicate that type 1 diabetes preceded neurological symptoms. In these cases, IVIG greatly improved the neurophysiological symptoms, but metabolic variables remained unaffected (7). As type 1 diabetes is a T cell-mediated disease in which the role of the humoral response in pathogenicity is still elusive (8), the effectiveness of IVIG is not self-evident. Indeed, IVIG treatment was evaluated as an immune therapy in type 1 diabetes but deemed unviable (9). The beneficial effect of IVIG treatment was unexpected but replicated (three times in total; **Figure 1**). During one of these remissions within 1 month of IVIG treatment, we were able to obtain evidence of immunoregulation of islet autoimmunity. Several mechanisms by which IVIG could affect the immune system have been identified that include activation of natural Tregs and anti-idiotypic antibodies present in IVIG (10).

Our observation that type 1 diabetes may be a relapsing/ remitting disease has significant therapeutic implications (2). Regulatory cytokine production during disease remittance suggests the presence of an antigen-specific regulatory compartment that can be therapeutically exploited. A relapsing/remitting model also implies a period where enough residual beta cell mass exists to restore beta cell function and glycaemic control. Finally, the notion that insulin insufficiency discords with beta cell mass begs reconsideration of the current use of beta cell function as a measure of efficacy for immune interventions aimed at protecting beta cells from autoimmune destruction.

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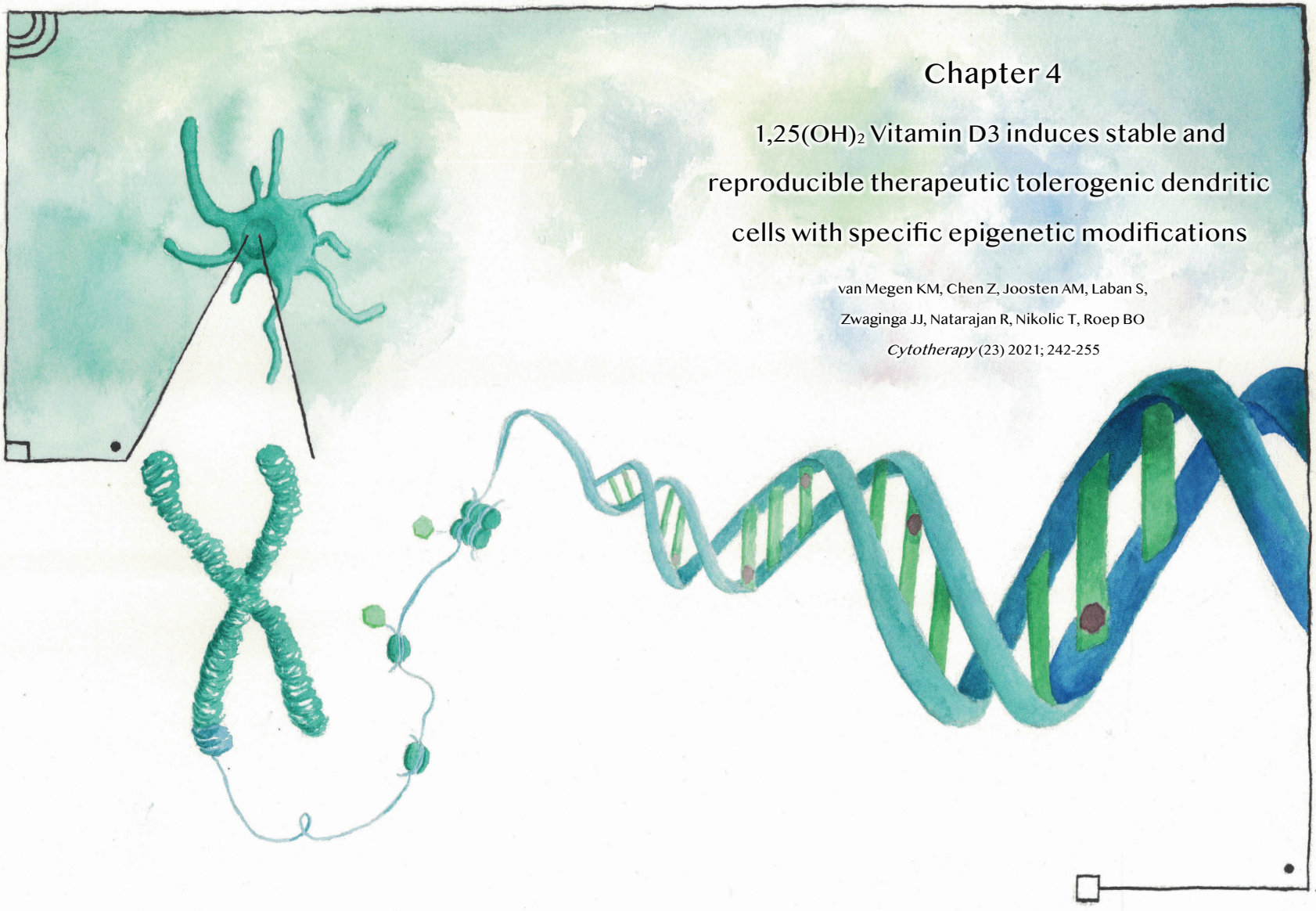
PART II

USING ANTIGEN-PRESENTING CELLS TO REINSTATE IMMUNE BALANCE IN TYPE 1 DIABETES



Chapter 4

$1,25(OH)_2$ Vitamin D3 induces stable and reproducible therapeutic tolerogenic dendritic cells with specific epigenetic modifications

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Abstract

Autologous, antigen-specific, tolerogenic dendritic cells (tolDCs) are presently assessed to reverse and possibly cure autoimmune diseases such as type 1 diabetes (T1D). Good Manufacturing Practice production and clinical implementation of such cell therapies critically depend on their stability and reproducible production from healthy donors and, more importantly, patient-derived monocytes. Here the authors demonstrate that tolDCs (modulated using 1,25-dihydroxyvitamin D3 and dexamethasone) displayed similar features, including protein, transcriptome and epigenome profiles, between two international clinical centers and between T1D and healthy donors, validating reproducible production. In addition, neither phenotype nor function of tolDCs was affected by repeated stimulation with inflammatory stimuli, underscoring their stability as semi-mature DCs. Furthermore, tolDCs exhibited differential DNA methylation profiles compared with inflammatory mature DCs (mDCs), and this was already largely established prior to maturation, indicating that tolDCs are locked into an immature state. Finally, approximately 80% of differentially expressed known T1D risk genes displayed a corresponding differential DNA methylome in tolDCs versus mDCs and metabolic and immune pathway genes were also differentially methylated and expressed. In summary, tolDCs are reproducible and stable clinical cell products unaffected by the T1D status of donors. The observed stable, semi-mature phenotype and function of tolDCs are exemplified by epigenetic modifications representative of immature-stage cells. Together, the authors' data provide a strong basis for the production and clinical implementation of tolDCs in the treatment of autoimmune diseases such as T1D.

Introduction

Dendritic cells (DCs) activate T cells to elicit an inflammatory or anti-inflammatory response, depending on whether the DC is inflammatory or tolerogenic (1). Tolerogenic DCs (tolDCs) are currently being evaluated as clinical cellular products for therapy in multiple autoimmune disorders, including type 1 diabetes (T1D) (2-8). T1D is a T-cell-mediated disease in which insulin-producing beta cells are attacked by autoreactive T cells (9). TolDCs can be generated in vitro from the peripheral blood of T1D patients by isolating and modulating monocytes with 1,25-dihydroxyvitamin D3 (VD3), followed by dexamethasone (10). TolDCs can subsequently be pulsed with disease-specific peptides to potentiate the capacity to reeducate the immune system in an antigen-specific fashion, which, in the case of T1D, can help to preserve beta cells (11).

Stability of a cellular phenotype or function could be supported by epigenetic regulation of gene expression (12). The field of epigenetics deals with heritable alterations in gene expression in the absence of changes in the underlying DNA sequence. Epigenetic status is maintained by several mechanisms, including DNA methylation (13-15). The authors produced tolDCs by treating monocytes with VD3, which acts by binding to the nuclear VD3 receptor. VD3 has long been linked to immunomodulation (16). Although there are data from various other cell types, the epigenetic effects of VD3 have not yet been explored in human DCs.

The authors found that, in human DCs, VD3 followed by dexamethasone significantly altered the expression of almost half of the transcripts of known T1D risk genes (17,18). In addition to the effect of VD3 on T1D risk genes, VD3 triggers metabolic changes with upregulation of glycolysis, which is essential for tolerogenic function (19). TolDCs modulate the immune system by secreting anti-inflammatory cytokines, such as IL-10, and by influencing other immune cells via cell surface markers. TolDCs have low T-cell stimulatory capacities, partly due to low expression of co-stimulatory molecules such as CD86, and are capable of inducing T regulatory cells (20,21). Moreover, tolDCs express lower levels of HLA-DR compared with inflammatory DCs, resulting in lower T-cell stimulatory capacity in a mixed lymphocyte reaction (MLR) (20). Because of these properties, tolDCs have been said to have a semi-mature phenotype (22,23). Immature DCs are not yet inflammatory, and maturation triggers an inflammatory machinery that grants mature DCs (mDCs) the co-stimulatory tools necessary for T-cell priming and activation (24-26). If arrested in this semi-mature stage, tolDCs would not be affected by further maturation challenges *in vivo*, securing their anti-inflammatory nature and legacy.

For clinical translation and utility of a cellular product, reproducibility of a stable and effective cell product from different donors is of the utmost importance. Ideally, this reproducibility in manufacturing should be achievable in multiple clinical centers. A safety

trial was conducted at Leiden University Medical Center (LUMC), Leiden, the Netherlands, evaluating tolDCs in T1D patients (D-Sense trial) (27), and presently, a phase 1b clinical trial is being set up at the City of Hope Medical Center (COH), Duarte, California, USA, to assess safety and feasibility in C-peptide-positive T1D patients.

In this study, the authors examined the stability of tolDCs by perturbing them with multiple inflammatory stimuli. In addition, the authors studied the reproducibility of tolDCs between two international production centers and between healthy subjects and T1D patients. Finally, the authors explored whether epigenetic modifications induced by VD3 may help to explain the observed stability of tolDCs.

Methods

Donor selection and database generation

Blood samples for tolDC cultures were taken from healthy blood donors and processed at either LUMC or COH. Samples from the D-Sense clinical trial were taken from T1D patients and produced at LUMC (27). All donors gave informed consent.

DC culture

DCs were cultured as described previously (28). In short, peripheral blood mononuclear lymphocytes were isolated from buffy coats collected from either healthy or T1D blood donors. CD14+ selection was performed with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and monocytes were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), glutamine and penicillin/streptomycin (Life Technologies), recombinant human IL-4 at 500 U/mL (Invitrogen, Breda, the Netherlands) and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) at 800 U/mL (Invitrogen) for 6 days. To induce tolDCs, clinical-grade VD3 at 10^{-8} M (32222-06-3; Dishman Carbogen Amcis, Veenendaal, the Netherlands) was added on day 0 and day 3. On day 3, dexamethasone at 10^{-6} M (Sigma-Aldrich) was also added to the tolDC culture. On day 3, culture medium was refreshed by discarding 50% of the medium and adding the same volume and twice concentrated IL-4 and GM-CSF to all cell cultures. On day 6, immature DCs were harvested and matured for 24-48 h by adding a cytokine mix, including GM-CSF, human recombinant IL-1β at 1600 U/mL, human recombinant IL-6 at 500 U/mL and human recombinant tumor necrosis factor alpha (TNF-α) at 335 U/mL (Miltenyi Biotec), and synthetic prostaglandin E2 at 2 μg/mL (Pfizer). Supernatant at day 8 was collected for further analysis of cytokine production. After maturation, DCs were phenotyped by flow cytometry, used for an MLR test, secondly matured or stored in liquid nitrogen. For second maturation with inflammatory stimuli, DCs were rested for 5 days in culture media supplemented with GM-CSF, after which a second round of maturation was performed. DCs were then

stimulated with the previously stated cytokine mix, lipopolysaccharide (LPS) (100 ng/mL) or CD40 ligation via co-culture with CD40 ligand (CD40L)-expressing L cells (0.5 x 10^6 DCs:0.2 x 10^6 L cells) for 24-48 h. For the second maturation experiments, cells were analyzed immediately after the first and second maturations.

Phenotype analysis

Unless stated otherwise, antibodies for phenotype analysis were purchased from BD Pharmingen (San Diego, CA, USA) and were the following: fluorescein isothiocyanate-conjugated HLA-DR, CD80, IgG2A, CD52 (Bio-Rad, Hercules, CA, USA) and IgG2B (Bio-Rad); phycoerythrin-conjugated CD1a, CD86, IgG1 and CD83 (Beckman Coulter, Brea, CA, USA); phycoerythrin-Cy7-conjugated CD14 (eBioscience, San Diego, CA, USA), ILT-3 (Beckman Coulter) and IgG1 (eBioscience); PercPCy5.5-conjugated CD209 and IgG2B; and allophycocyanin-conjugated IgG1, CD3, CD25, PD-L1 (eBioscience) and CD40 (eBioscience). DCs were incubated with a mix of monoclonal antibodies for 30 min on ice. Cells were washed in fluorescence-activated cell sorting (FACS) buffer containing 1% fetal bovine serum and 0.05% sodium azide (Sigma-Aldrich) and analyzed using FACSCanto or Fortessa (BD Biosciences). Data were analyzed using FACSDiva 8 (BD Biosciences) and FlowJo 10 software (Ashland, Oregon, USA).

Cytokine analysis and MLR

After the culture period, supernatants from mDCs were harvested and analyzed for cytokine analysis with the nine-plex Bio-Plex Pro human cytokine Th1/Th2 assay Luminex kit (Bio-Rad) according to the manufacturer's protocol. In parallel, the cells were analyzed for T-cell stimulatory capacity in an MLR. The cells were harvested and replated in a flat bottom 96-well plate in different concentrations in triplicate in Iscove's Modified Dulbecco's Medium with 10% inactivated human serum (Sanquin, Amsterdam, the Netherlands). Allogeneic CD4+ T cells were obtained from HLA-typed peripheral blood mononuclear lymphocytes using the Dynabeads untouched CD4 T-cell kit (Invitrogen) according to the manufacturer's protocol. Next, 1 x 10^4 allogeneic CD4 + T cells were added to the wells, and after 4 days of culture they were pulsed overnight with [^{3}H]-thymidine 0.5 µCi/well. Thymidine incorporation was measured using a liquid scintillation counter (PerkinElmer, Groningen, the Netherlands). The counts per minute of the tolDC condition were divided by the counts per minute of the mDC condition (positive control) and multiplied by 100 to provide the stimulation index (SI). The change in T-cell stimulation from the first maturation for the additional inflammatory stimuli was calculated by the delta SI.

$$SI(\%) = (CPMtolDC/CPMmDC) * 100$$

Delta SI (change from first maturation) = SI second maturation — SI first maturation.

Metabolic analysis

The XF^{e}96 extracellular flux analyzer (Seahorse Bioscience, MA, USA) was used to measure the mitochondrial oxygen consumption rate (mpH/min) and extracellular acidification rate (mpH/min). On day 6, immature DCs were harvested and matured with the previously mentioned cytokine mix for 24-48 h in a 96-well Seahorse plate at 4 x 10^4 cells per well. After maturation, the plate was spun down with slow acceleration, and break off settings and cells were carefully washed. Next, 5 µg/mL human recombinant soluble CD40L (PeproTech, Rocky Hill, NJ, USA) was added for 18 h. After spinning down the plate with slow acceleration and break off settings, DCs were carefully washed three times in either glycolysis stress test assay medium (Dulbecco's Modified Eagle's Medium base, 2 mM L-glutamine, pH 7.35) or mitochondrial stress test medium (Dulbecco's Modified Eagle's Medium base, 2 mM L-glutamine, 1 mM pyruvate, 25 mM glucose, pH 7.35) and incubated in a non-carbon dioxide incubator at 37°C for 1 h. The following compounds were used for the glycolysis and mitochondrial stress tests: 10 mM glucose, 1.7 µM oligomycin, 50 mM 2-deoxy-D-glucose, 0.5 M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, 0.5 µM rotenone and 0.5 µM antimycin A. The plate was analyzed on a XF^{e}96 extracellular flux analyzer (Seahorse Bioscience) using the standard stress test templates. After the assay, the plate was collected and analyzed for cell number using a Celigo cytometer (Nexcelom Bioscience, San Diego, CA, USA). Oxygen consumption rate and extracellular acidification rate values were normalized to cell number.

RNA and genomic DNA preparations

A total of 11 donors were used for these studies, of which three DC donors came from COH and three from a previous study conducted at LUMC (17), and the other five donors were from the authors' D-Sense clinical trial conducted at LUMC. DNA and RNA from these samples were extracted using a Quick-DNA/RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol. Only RNA with an RNA integrity number $\geq$8 was used in the polyA sequencing library preparation method for RNA sequencing (RNA-seq) (Illumina, San Diego, CA, USA). The genomic DNA was analyzed for DNA methylation levels by the Infinium MethylationEPIC array (Illumina) according to the manufacturer's instructions. Both assays were performed by the genomics core at COH.

RNA-seq library preparation and sequencing with Illumina HiSeq 2500

RNA-seq libraries were prepared with a messenger RNA HyperPrep kit (KR1352; Kapa Biosystems) according to the manufacturer's protocol. Briefly, 250 ng of total RNA from each sample was used for polyA RNA enrichment. The enriched messenger RNA underwent fragmentation and first strand complementary DNA (cDNA) synthesis. The combined second cDNA synthesis with 2'-deoxyuridine 5'-triphosphate and A-tailing reaction generated the resulting double-stranded cDNA with deoxyadenosine

monophosphate to the 3' ends. The barcoded adaptors were then ligated to the double-stranded cDNA fragments. A 12-cycle polymerase chain reaction was performed to produce the final sequencing library. The libraries were validated with the Bioanalyzer DNA high sensitivity kit (Agilent) and quantified with Qubit. RNA-seq libraries were sequenced on the Illumina HiSeq 2500 using an SR v4 kit with the single read mode of 51 cycles of read1 and seven cycles of index read. Real-time analysis 2.2.38 software was used to process the image analysis and base calling.

RNA-seq data analysis

Raw RNA-seq reads were trimmed to remove sequencing adapters using Trimmomatic (29) and polyA tails using FASTP (30). The processed reads were aligned to the human genome (hg19) using STAR 020201 software (31). HTSeq 0.6.0 software (32) was then applied to generate the count matrix on Reference Sequence (RefSeq) genes with default parameters. The resulting counts were normalized using the trimmed mean of M-values method provided by the edgeR package in R (33) to obtain normalized expression values. For between cell type comparison, general linear models were applied to identify DEGs between two specific cell types using the trimmed mean of M-values normalized expression level as dependent variable and cell type as independent variable, adjusting for disease status and location for each sample. For comparison between different locations (COH versus LUMC) or health status (T1D versus healthy) within one specific cell type, similar models were used with location/ health as dependent variable, adjusting for health or location, respectively. Genes with a false discovery rate <0.05 and a fold change (FC) >2 or <0.5 were considered significantly upregulated and downregulated genes, respectively.

Illumina Infinium HD methylation assay

The genomic DNA samples were treated with bisulfite using the EZ DNA methylation kit from Zymo Research with the alternative incubation condition for the Illumina Infinium HD methylation assay according to the manufacturer's protocol. Briefly, the bisulfite-converted DNA was denatured with 0.1 N sodium hydroxide and amplified for 20-24 h in the 37°C hybridization chamber to produce a sufficient DNA sample. The amplified DNA was enzymatically fragmented at 37°C for 1 h and precipitated for 30 min at 4°C. To hybridize the DNA onto Illumina BeadChips, the precipitated DNA was resuspended using RA1 solution according to the manufacturer's protocol, and the suspended DNA with an appropriate volume was loaded onto the eight-sample Infinium MethylationEPIC BeadChip (Illumina). The hybridization was performed for over 16 h within a 24-h period. After washing the hybridized BeadChip, the primers hybridized to DNA were extended and incorporated with the labeled nucleotides for the multilayer staining process. The image acquisition was carried out using the Illumina iScan System.

DNA-me profiling and data analysis

After quality controls with Illumina's GenomeStudio, data were pre-processed using R package minfi. Specifically, background correction was followed by subset quantile within array normalization and quantile normalization. DNA methylation level, or beta value, was then generated for each CpG site and each sample. CpG sites with detection $P \geq 0.01$ in at least one sample were excluded. To identify differentially methylated CpGs (DMCs) among groups (mature tolerogenic, immature tolerogenic, mature inflammatory, immature inflammatory), multiple linear regressions were performed using beta values as response variable and group as explanatory variable, adjusting for patients. To identify DMCs among different sites, sites were used as explanatory variable without adjusting for patients. The following criteria were used to select the significant DMCs: (i) P 0.01, (ii) difference $\geq$0.15 and (iii) at least one group with mean methylation level $\geq$0.25. The significance level of difference between two groups genome-wide was visualized using a Manhattan plot generated using R package qqman v.0.1.4. Hierarchical clustering was performed combining all DMCs with Pearson correlation as distance matrix and average linkage using Cluster 3.0. A heatmap was generated using Java TreeView 1.1.6r4.

The significant DMCs were merged into differentially methylated regions (DMRs) if their difference was $\leq$200 bp. Multiple linear regressions were performed on each of these DMRs using beta value as response variable and group as explanatory variable, adjusting for patient and CpG position, to summarize region-level difference.

CpGs and DMRs were annotated to genomic regions (transcription start site [TSS] 200 [200-bp upstream region of TSS] and TSS1500 [-1500 bp to approximately -200 bp relative to TSS], 5' untranslated region, coding exon, intron and 3' untranslated region) relative to RefSeq genes (hg19; University of California Santa Cruz Genome Browser) based on their location. CpGs or DMRs not located in any of the aforementioned regions were considered to locate in intergenic regions. Ingenuity pathway analysis (IPA) (Qiagen) or Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis was applied to the annotated genes of DMCs or DMRs to obtain enriched biological processes or pathways. De novo motif analysis was performed on DMRs between mature tolDCs (mtolDCs) and mDCs using RSAT Metazoa Regulatory Sequence Analysis Tools (http://rsat.sb-roscoff.fr/peak-motifs_form.cgi) to detect enriched motifs using $\pm$250-kb regions of the least differentially 5000 CpGs as background. The top identified significantly enriched motifs were then queried against core non-redundant vertebrates (2018) in JASPAR to identify any known transcription binding sites.

Integration analysis of DNA-me and gene expression

For each DEG identified between mtolDCs and mDCs, DMCs located in eight non-overlapping genomic regions relative to genes were identified. The regions included

promoter (2.5 kb upstream of TSS), gene body, approximately 0-5 kb upstream of promoter, approximately 0-5 kb downstream of gene body, approximately 5-50 kb upstream of promoter, approximately 5-50 kb downstream of gene body, approximately 50-500 kb upstream of promoter and approximately 50-500 kb downstream of gene body. The gene lists containing the differentially methylated locus (DML) in each of these regions were also uploaded to IPA for Gene Ontology analysis.

Gene Ontology analysis

Pathway analysis was conducted using DAVID 6.8 (34,35). GSEAP-reranked analysis was performed using the Gene Set Enrichment Analysis desktop program in Java (36,37) based on a ranked list of whole genes according to their log2 FC and P values.

Analysis on T1D risk genes

T1D risk genes were identified as those genes located on the T1D-associated regions provided by www.t1dbase.org. Specifically, except for one region, all the regions (based on hg38 human genome assembly) were lifted over to hg19 assembly using the University of California Santa Cruz LiftOver tool (http://genome.ucsc.edu/cgi-bin/ hgLiftOver) to render them consistent with the genome assembly used by the MethylationEPIC array (Illumina). The 441 RefSeq genes located in these 56 regions were considered potential T1D risk genes, and among these, 62 located in 28 regions showing differential expression between mtolDCs and mDCs were retrieved. DMCs located in these 62 T1D risk DEGs were further identified as described previously. The T1D DEGs and their associated DMLs located within 5-kb flanking regions are shown as circular plots using R package circlize 0.4.3.

Statistical analysis

Data were analyzed with Prism 7 (GraphPad Software). Either an unpaired Student's t-test or analysis of variance was used to test statistical significance. $P < 0.05$ was considered significant.

Results

TolDCs retain semi-mature immunophenotype after stimulation with LPS, inflammatory cytokines and CD40L

All human experiments were performed following informed consent and approval from the institutional review boards, in accordance with approved protocols, at both clinical centers. Cells were processed at either LUMC or COH. DCs were derived from purified monocytes from buffy coats of anonymous healthy donors and cultured in six-well plates. Monocytes for the D-Sense clinical trial were obtained by apheresis procedures in T1D

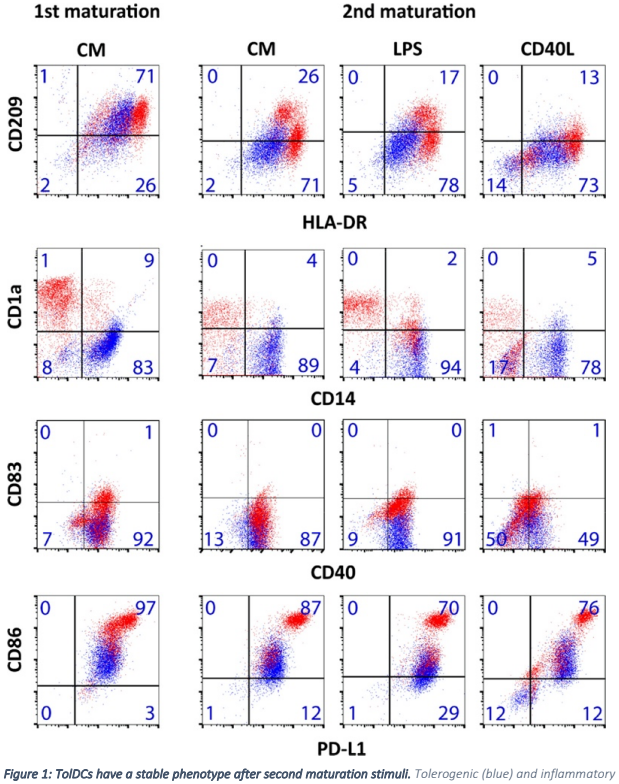
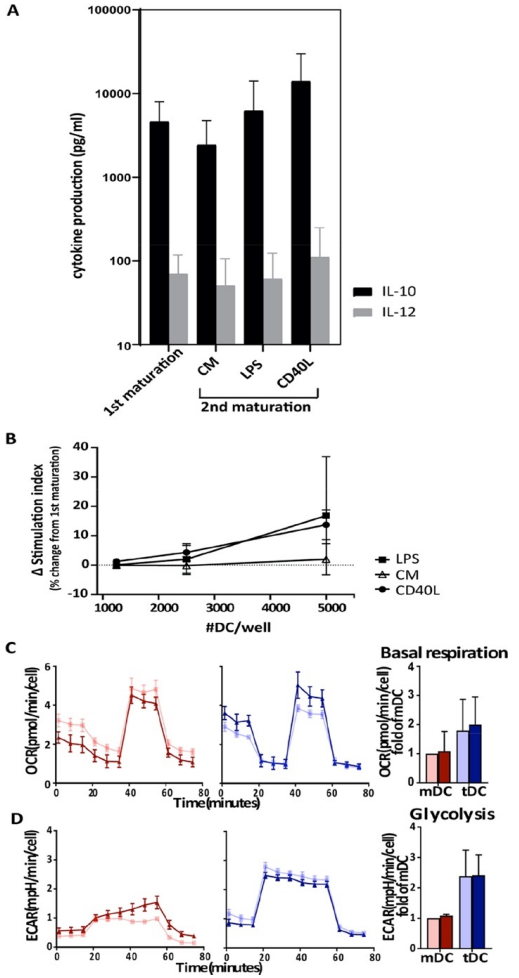


Figure 1: TolDCs have a stable phenotype after second maturation stimuli. *Tolerogenic (blue) and inflammatory (red) DCs were first matured with CM and analyzed by flow cytometry. Subsequently, these cells were rested for 5 days and perturbed with another round of maturation stimuli, such as CM, LPS and CD40L, and analyzed. Phenotypic markers were analyzed by flow cytometry, and quadrant gates were set on the corresponding isotype controls. Percentage positivity per gate for tolDCs is noted in each box. Plots are representative of three independent experiments. The phenotype after the 5-day rest period was similar to the first maturation and not shown. With the exception of decreased expression of CD209 upon second maturation stimuli in both tolerogenic and inflammatory DCs, no changes in phenotype were noted upon second maturation. In the case of CD40L-stimulated cells, double-negative events in the lower left quadrant represent CD40L-expressing fibroblasts. ILT-3 was not significantly different between tolerogenic and inflammatory DCs (5.1 ± 12.0% versus 9.1 ± 15.4%, respectively, n = 33 donors, P = 0.18). CM, cytokine mix.*



patients and cultured in culture bags (27). All standard operational procedures and reagents in the manufacturing process of DCs were similar, regardless of processing location or clinical status. In short, monocytes were isolated from peripheral blood and treated with GM-CSF and IL-4 to produce inflammatory DCs, and in the case of tolDCs, the culture medium was additionally supplemented with VD3 and dexamethasone. Maturation of inflammatory and tolDCs was achieved by treatment with a cytokine mix (IL-6, TNF, IL-1β and prostaglandin E2).

Upon injection into a patient, mature tolDCs may encounter inflammatory stimuli *in vivo* that could affect their phenotype and function. To assess whether tolDCs are locked into a definitive semi-mature state, mature tolDCs were restimulated with inflammatory stimuli, and their phenotype and function were subsequently tested. Specifically, after the first cytokine-stimulated maturation and a rest period of 5 days, another inflammatory stimulus with LPS, CD40L or the same cytokine mix followed (**Figure 1**). The first cytokine-stimulated maturation induced an increase in HLA-DR, CD83 and CD86 expression in inflammatory DCs (mDCs), segregating mature tolDCs from mDCs. After the restimulation, tolDCs largely retained the phenotype acquired in the first step; in particular, they remained HLA-DR^{low}, CD14^{+}, CD1a^{-}, CD83^{-}, CD86^{low} and PD-L1^{+} and consistently distinct from inflammatory DCs stimulated in parallel, as examined by flow cytometry. The only exception was CD209 (also called DC-specific ICAM-3-grabbing non-integrin) expression, which decreased upon restimulation in both tolDCs and inflammatory DCs (**Figure 1**). CD209 is known to recognize ICAM-3 on T cells or ICAM-2 on endothelial cells and thereby has a role in trafficking and T-cell interactions (38,39).

Figure 2: TolDCs have stable function and metabolism after repeated inflammatory stimuli. *Tolerogenic and inflammatory DCs were matured with CM. Subsequently, these cells were perturbed with in vivo-simulating stimuli, such as CM, LPS and CD40L.* ***(A)*** *In vivo simulations with CM, LPS or CD40L did not change IL-10 or IL-12 cytokine concentrations in tolDCs (n = 4). Error bars show SD.* ***(B)*** *Relative change in capacity to stimulate T cells (SI) between first maturation and in vivo inflammatory simulations calculated as described earlier. No significant increase in immunogenicity of tolDCs was observed after additional inflammatory stimuli compared with first maturation. Graphs are representative of three independent experiments.* ***(C,D)*** *Seahorse analysis was performed to assess the effect of in vivo-simulating stimulus CD40L (in dark blue and dark red for tolerogenic and inflammatory DCs, respectively) on DC metabolism. DCs that received only the first maturation are in light blue for tolDCs and light red for mDCs. An oxygen consumption (C) and glycolysis stress test (D) was performed (n = 3). OCR and ECAR normalized to cell number are shown with SEM. To the right of the graphs (C,D), bar graphs of summary data of three independent donors show basal respiration and glycolysis calculated from graph data, with error bars showing SD. CD40L did not significantly change basal respiration or glycolysis. CM, cytokine mix; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; SD, standard deviation; SEM, standard error of the mean; SI, stimulation index.*

TolDCs retain semi-mature functional aspects and metabolism after additional inflammatory stimuli

In addition to flow cytometry, cytokine and functional analyses were conducted. None of the repeated inflammatory stimuli changed the cytokine profile of tolDCs (**Figure 2A**). Next, the authors assessed the T-cell stimulatory capacity of DCs in an MLR test in which DCs were co-cultured with allogeneic CD4+ T cells and proliferation of T cells was measured. Mature inflammatory DCs elicited a strong allo-reaction, whereas tolDCs only minimally stimulated T cells to proliferate in an MLR (3.5 $\pm$ 2.8% of T-cell stimulation by mDCs). Repeated stimulation did not significantly change the low T-cell stimulatory capacity of tolDCs (**Figure 2B**).

Recently, the authors showed that distinct metabolism is another functional marker for tolDCs (19). Since CD40 ligation on DCs by T cells directly activates DCs in the context of antigen presentation on HLA class II (1), the authors deemed CD40L the most relevant physiological stimulus for also studying DC metabolism in a Seahorse assay. In concordance with the authors' previous study (19), tolDCs elicited higher oxygen consumption, glycolysis and glycolytic capacity than mDCs (**Figure 2C,D**; also see **Suppl. Figure 1**). CD40 ligation did not change oxygen consumption rate, as basal respiration and maximal respiration were unaltered in tolDCs and mDCs. In terms of glycolysis, CD40 ligation sensitized mDCs to oligomycin treatment, which increased extracellular acidification rate, quantified as a significant increase in glycolytic capacity (see **Suppl. Figure 1**). TolDCs, however, remained insensitive to oligomycin treatment and did not exhibit changes in any of the glycolysis parameters upon CD40L treatment.

In summary, tolDCs displayed stable phenotype, function and metabolic activity even after repeated inflammatory stimuli. Therefore, tolDCs appear to be arrested in a semi-mature state.

TolDCs display a differential transcriptome compared with inflammatory DCs that is unaffected by health status or production location

Stability of a cellular product, including reproducible production between different international centers and between healthy subjects and T1D patients, is important for its implementation in the clinic. Therefore, the authors produced tolDCs from healthy subjects and T1D patients that passed validated quality control criteria (low CD86 expression, high CD52 expression (27)) in two international production centers. Subsequently, for more in-depth analysis, a transcriptome study was conducted comparing gene expression by RNA-seq between DCs produced at LUMC and COH. Finally, immature (day 6 of culture) and mature (day 8 of culture) DCs were compared to assess whether tolDCs maintained a stable semi-mature transcriptome.

In agreement with the authors' previous studies, mature tolDCs showed increased expression of genes involved in glycolysis and oxidative phosphorylation and decreased expression of genes involved in interferon gamma (IFN-γ) signaling, unfolded protein response and antigen processing and presentation (see supplementary **Figure 2A**) (17). DAVID pathway analysis confirmed that mature tolDCs displayed decreased cell activation pathways, in particular T-cell activation and response to cytokines, compared with mDCs.

Unsupervised hierarchical clustering of 10,854 expressed genes showed discrete cell types in all samples regardless of production site or health status (**Figure 3A**). When examining the number of differentially expressed genes (DEGs) (**Figure 3B**; also see supplementary **Figures 3, 4**), most were found between mature tolerogenic and inflammatory DCs (1663 upregulated and 1333 downregulated genes), with the fewest being found between immature tolerogenic and inflammatory DCs (760 upregulated and 795 downregulated genes). The effect of maturation was more prominent in inflammatory DCs, as they displayed more DEGs between the immature and mature states than tolDCs (1540 upregulated and 1557 downregulated genes versus 1119 upregulated and 1275 downregulated genes, respectively) (see **Suppl. Figures 5,6**), suggesting that mature tolDCs could be more similar to their immature state than inflammatory mDCs are to their immature state.

In line with this, mature tolDCs showed increased expression of markers associated with an immature phenotype compared with mDCs (*CD52*, C-C chemokine receptor type 1 [*CCR1*], *CCR5*, low affinity immunoglobulin gamma Fc region receptor III-A and mannose receptor) (see supplementary Table 1). In addition, mature tolDCs clustered relatively closer to their immature state than mDCs did in a principal component analysis (**Figure 3C**). Furthermore, they showed more homogeneity within the immature and mature states than did inflammatory DCs. Besides, clustering was primarily based on cell type, rather than health status or manufacturing center (**Figure 3C**; also see **Suppl. Figures 7, 8**). Indeed, only a few genes in mature tolDCs were differentially expressed in different centers and between healthy subjects and T1D patients (**Figure 3D**) compared with the large number of DEGs in the cell type comparisons (**Figure 3B**).

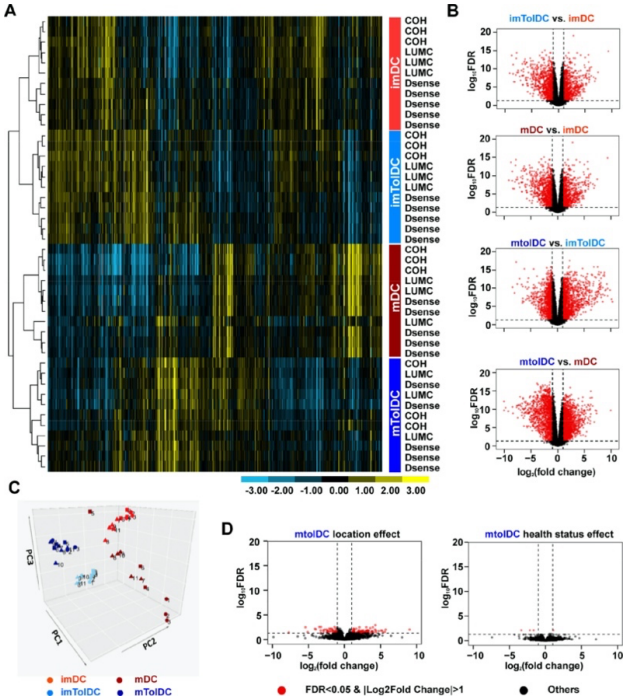


Figure 3: Transcriptomic analysis of tolDCs reveals that they are unaffected by location or T1D status and are more similar to their immature state than inflammatory DCs. *RNA was isolated at the immature and mature stages of DC production, and RNA-seq was performed on Illumina HiSeq 2500. Samples clustered based on cell types, which are shown in the colored box to the right of the heatmap. Inflammatory imDCs are shown in light red, mDCs in dark red, imtolDCs in light blue and mtolDCs in dark blue.* ***(A)*** *Unsupervised hierarchical clustering of approximately 10% of 10,854 expressed genes (selected from the 21,121 genes using criterion RPKM > 1 in at least four samples). Each row represents one sample, which is labeled by location to the right of the heatmap, with the color of the label designating the cell type. Data show that samples clustered on cell type rather than location or health status.* ***(B)*** *Volcano plots depict DEGs in different cell type comparisons. For all volcano plots, red dots represent significant genes with an FC > 2 and FDR < 0.05, and black dots represent all other expressed genes. For a zoomed in view of volcano plots and identities of DEGs, see supplementary Figures 6–11.* ***(C)*** *PCA plot of all samples. Squares indicate samples from COH, circles from LUMC and triangles from D-Sense.* ***(D)*** *Volcano plots comparing location and health status in mtolDCs. FDR, false discovery rate; imDC, immature DC; imtolDC, immature tolDC; mtolDC, mature tolDC; PCA, principal component analysis.*

In summary, the authors' transcriptomics data corroborated previous findings that mature tolDCs display a reduced capacity to stimulate T cells while having increased metabolic pathways. In addition, the authors' current study demonstrated that mature tolDCs were more similar to their immature state than their inflammatory counterparts. Overall, tolDCs portrayed some degree of stability in gene expression associated with immature DCs and were largely unaffected by manufacturing location or T1D status.

The differential DNA methylation profile of tolDCs compared with inflammatory DCs is reached at the immature state of the cells and remains unaffected by production site or T1D status

Stability may be explained by epigenetic modifications like DNA methylation (40). The authors therefore performed DNA methylation profiling with Illumina human MethylationEPIC arrays on DNA samples isolated from the same samples as those used for RNA-seq. With this, the authors identified differentially-methylated CpGs (DMCs) in response to VD3 modulation as described earlier. Data analyses showed that donor health status as well as production site had minimal effect on DNA methylation in mature tolDCs (26 and 13 DMCs, respectively) (see supplementary Table 2). However, a large number of DMCs were noted between immature tolerogenic and inflammatory DCs (2463 DMCs) (**Figure 4A,B**). After maturation, the number of DMCs between mature tolerogenic and inflammatory DCs increased further to 3457, the majority of which were retained from the immature state (2217 DMCs) (see **Suppl. Figure 9**). This suggests that most of the DNA methylation modifications seen after VD3 treatment were already present at the immature state. Indeed, in the heat-map of DMCs (**Figure 4C**), the two most distinct clusters are tolerogenic versus inflammatory DCs, rather than immature mDCs versus mDCs. Furthermore, mature tolDCs showed fewer DMCs between their immature states compared with inflammatory DCs (384 and 742 DMCs, respectively) (**Figure 4A**). Specifically, 475 CpGs were demethylated upon inflammatory DC maturation but remained unchanged upon tolDC maturation (**Figure 4C**). These were enriched for genes associated with lymphocyte differentiation and leukocyte cell-to-cell adhesion (Q < 0.05) (see supplementary Table 3). Examples of genes in these pathways were *CD86, CD25, IL23R,* TNF super family member 4, *IL7R, CCR6* and nuclear factor κ light chain enhancer of B cells (*NFκB*) subunit *RELB*.

DNA demethylation can be caused by ten-eleven translocation (TET) enzymes and changes in de novo DNA methylation by DNA methyltransferases (41). Although the expression of *TET1* was undetected by RNA-seq, *TET2* was upregulated (FC = 1.50, Q = 0.0050) and *TET3* was downregulated (FC = -1.73, Q < 0.0001), whereas DNA methyltransferase 3 α was upregulated in mtolDCs compared with mDCs (FC = 1.73, Q

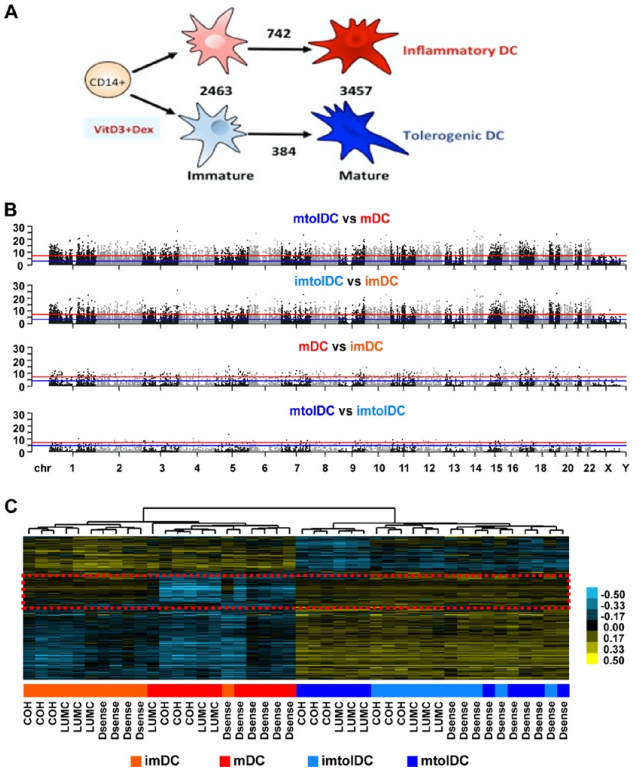


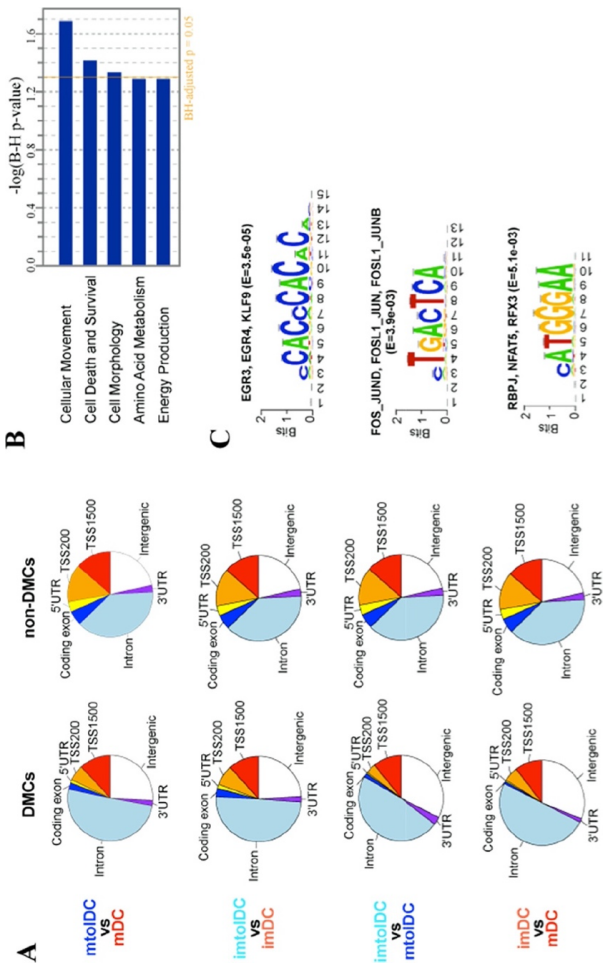
Figure 4: The differential DNA methylation profile of tolerogenic compared with inflammatory DCs is present at the immature state and is unaffected by location or T1D status. *Genomic DNA was isolated at the immature and mature stages of DC production and subjected to DNA methylation profiling with Infinium MethylationEPIC arrays. Inflammatory imDCs are shown in light red, mDCs in dark red, imtolDCs in light blue and mtolDCs in dark blue.* ***(A)*** *Schematic representation of mDC versus tolDC culture and numbers of DMCs between different cell type comparisons.* ***(B)*** *Manhattan plots depicting the DNA methylation difference between different cell types, as indicated above the plot, across the human hg19 genome. Each dot represents one CpG, whose genomic location is represented by the x-axis and significance level in logarithm format by the y-axis. The red line represents Bonferroni-adjusted P 0.05, and the blue line represents FDR at 5%. Dots located above the lines are considered*

<0.0001). This is in line with the authors' finding that tolDCs showed higher DNA methylation levels, as seen in the heatmap, compared with inflammatory DCs. Indeed, 150 hypermethylated and only 37 hypomethylated regions were found in mature tolerogenic compared with inflammatory DCs.

Examining the genomic location of DMCs relative to RefSeq genes, the authors found they are mainly located in introns and intergenic regions, with around 25% of DMCs in coding exons and up to 1500 bp upstream of a TSS (**Figure 5A**). IPA of genes containing differentially methylated regions (DMRs) (including 150 hypermethylated regions and 37 hypomethylated regions) in promoter or gene bodies revealed cellular movement, cell death and survival, cell morphology, amino acid metabolism and energy production as the most enriched pathways ($P < 0.05$) (**Figure 5B**). In line with this, the top enriched biological processes identified on genes with DMRs were positive regulation of actin filament polymerization and regulation of cell shape (false discovery rate <1%) (see supplementary Table 4). Furthermore, these DMRs were enriched at binding motifs of transcription factors associated with inflammatory genes (*JUND, JUN, JUNB, FOS-Like1(FOSL1), FOS, Early growth response gene 3 (EGR3),* Kruppel Like Factor 9 (*KLF9*) which were all upregulated in mature tolDCs compared with inflammatory DCs (**Figure 5C**).

In summary, mature tolDCs have a differential DNA methylation profile compared with mature inflammatory DCs, and this is mostly already present at the immature state. TolDCs retain a hypermethylated state after maturation, whereas inflammatory DCs become demethylated upon maturation. Primarily, genes involved in eliciting an inflammatory immune response are associated with these regions of DNA demethylation in inflammatory DCs. In general, differences in methylation level between mature tolDCs and inflammatory DCs were enriched in pathways involved in cell morphology and movement. Finally, health status and production site had a negligible effect on tolDC DNA methylation.

(cont.) DMCs at corresponding confidence level. ***(C)*** *Heatmap depicting all DMCs identified in at least one comparison shown in B after unsupervised hierarchical clustering analyses. Each row represents one DMC, and each column represents one sample. Blue indicates DNA methylation below the average of all samples, whereas yellow indicates DNA methylation above the average, with the intensity level shown in the color bar. Each sample's group information is presented using a colored box below the heatmap, with color definitions indicated in the legend at the bottom of the panel and clinic locations where each sample was obtained indicated at the bottom of the heatmap. The red dashed box indicates a region of interest further analyzed in supplementary Table 8. Dex, dexamethasone; FDR, false discovery rate; imDC, immature DC; imtolDC, immature tolDC; mtolDC, mature tolDC.*

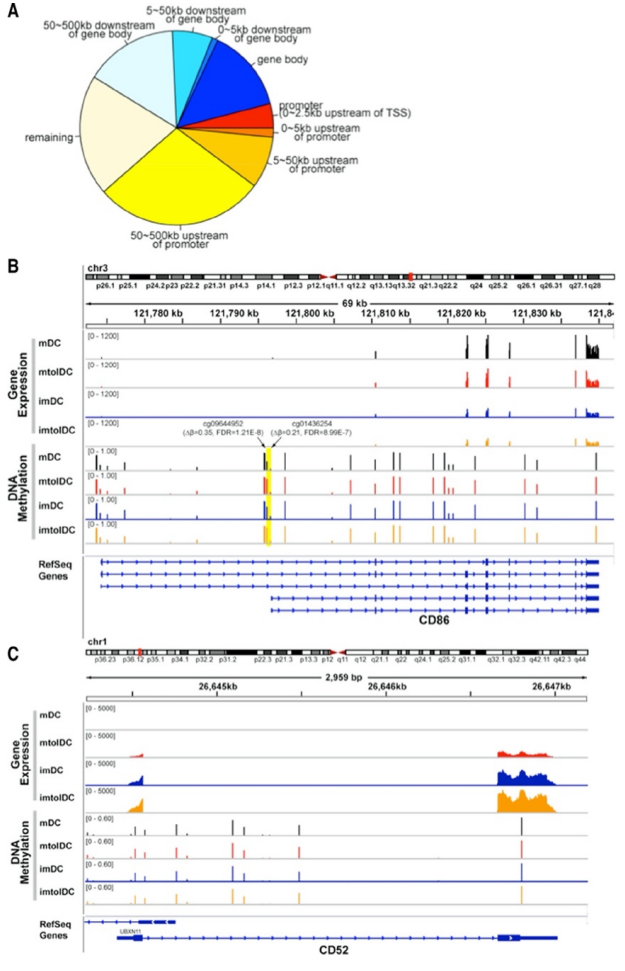


DEGs associated with DMLs between mature tolerogenic and inflammatory DCs are enriched in immune response and cellular movement pathways

DEGs were aligned with DNA methylation data from both mature tolerogenic and inflammatory DCs. Approximately 80% of DEGs had at least one DMC within 500 kb downstream of their gene body or upstream of their promoter (**Figure 6A**). Around 20% of DMCs were located directly in the promotor or gene body of the DEG. IPA on genes whose promoters contain DMCs showed that the top canonical pathways were granulocyte adhesion and diapedesis and vitamin D receptor/retinoid X receptor activation, whereas the top upstream regulators were progesterone receptor, jagged canonical notch ligand 2 and NFκB ($P < 0.0001$). The top upstream regulators, TNF, IL-13 and IFN-γ, were shared between the DEGs containing DMCs in promotors and gene bodies, whereas CD40L was specifically enriched in DMCs of the latter ($P < 0.0001$). In terms of molecular and cellular functions, both IPAs of DMCs in gene promotors and bodies revealed cellular movement, cell death and survival, cell-to-cell signaling and interaction and cellular development as enriched pathways ($P < 0.01$).

The authors also identified 121 DEGs containing multiple DMCs in their promoters (approximately 0-2.5 kb upstream of TSS). Among them were multiple DEGs involved in free fatty acid metabolism. Acyl-CoA thioesterase 7, for instance, had five DMCs in its promotor region, whereas acyl-CoA synthetase long chain family member 1 had three and solute carrier family 27 member 3 had one, and their expression was upregulated in tolDCs compared with mDCs. The chemokines, chemokine ligand 24 (*CCL24*) and *CCL13*, had two DMCs in their promotor as well and were upregulated and downregulated, respectively. Another immunological mediator, interleukin 1 receptor antagonist (*IL1RN*), which is a decoy protein for the IL-1 receptor, was strongly upregulated in mature tolDCs (log2FC = 2.51, Q = 0.0003) and had four DMCs in its promotor region compared with mature inflammatory DCs. In terms of the release criteria for the D-Sense clinical trial, *CD86* had significantly decreased expression (log2FC = -0.49, Q = 0.009), whereas *CD52* was highly upregulated (log2FC = 4.48, Q < 0.0001) in tolDCs versus mDCs. Interestingly, two highly significantly hypermethylated loci (cg01436254 and cg09644952) were identified in the proximal promoters of two shorter isoforms of CD86 (NM_176892 and

Figure 5: DMC and region analyses between tolerogenic and inflammatory DCs. (A) *Pie charts summarizing the genomic location of DMCs relative to RefSeq genes and other non-DMCs covered by MethylationEPIC array. CpGs were annotated to one of the following regions related to RefSeq genes: coding exon, 5'UTR, TSS200 (200 bp upstream of TSS), TSS1500 (1500 bp upstream to 200 bp upstream of TSS), 3'UTR and intron. CpGs not located in any of these regions are considered intergenic.* ***(B)*** *Bar plot of the top enriched biological processes identified on DMRs between mtolDCs and mDCs using IPA. The y-axis represents B-H-adjusted P values in log-transformed format.* ***(C)*** *De novo motif analysis followed by JASPAR vertebrate motif database query using DNA sequences at DMRs. Transcription factors whose binding motifs matched the motifs identified by de novo motif analysis are shown on top of the motif. B-H, Benjamini–Hochberg; imDC, immature DC; imtolDC, immature tolDC; mtolDC, mature tolDC; UTR, untranslated region.*



NM_006889) in mature tolDCs versus inflammatory DCs, whereas *CD52* had one DMC in the 500-kb flanking region of its TSS (**Figure 6B,C**). Moreover, the DNA methylation levels at cg01436254 in tolDCs were very similar to the levels seen in immature tolDCs and immature mDCs. In summary, the majority of genes differentially expressed between tolerogenic and inflammatory DCs were associated with DMCs, among which many are important for tolDC function.

The majority of T1D risk genes differentially expressed between tolerogenic and inflammatory DCs are associated with DMLs

The authors previously reported that VD3 alters the expression of approximately 30% of T1D risk genes in DCs (17). Here the authors found that 62 out of 198 (31%) expressed genes located in T1D-associated regions (T1D risk genes) were differentially expressed (including 35 upregulated and 27 downregulated genes) in tolerogenic versus inflammatory DCs (**Figure 7**). This was corroborated by the observation that the T1D Kyoto Encyclopedia of Genes and Genomes gene set that consists of 43 genes associated with T1D was significantly downregulated when comparing immature tolerogenic with inflammatory DCs as well as mature tolerogenic with inflammatory DCs (see **Suppl. Figure 2A,B**). Out of the 62 identified T1D risk DEGs, 52 genes (84%) had DMLs within the 500-kb flanking region of the TSS. Of these, 12 genes (19%) contained DMCs at their gene bodies and/or in the 5-kb flanking region. These included eight downregulated genes (Cytotoxic T-Lymphocyte Associated Protein 4 (*CTLA4*), *C-Type Lectin Domaine Family 2 Member D (CLEC2D), CLEC16A, IL2RA, Cyclin Dependent Kinase 2 (CDK2), RAB5B, Class II Major Histo-compatibility Transactivator (CIITA), IKAROS Family Zinc Finger 1 (IKZF1)*) and four upregulated genes (*CCR1, CLN3, Apolipoprotein B Receptor (APOBR), T cell Activation RhoGTPase Activating protein (TAGAP)*) in mature tolDCs versus mDCs (**Figure 7**). The most significant DMCs for each of these genes were hypermethylated in mtolDCs versus mDCs (**Figure 7**). In summary, VD3 alters the expression of T1D risk genes in DCs, and the altered expression of at least a subset of these genes might be regulated by DNA methylation, revealing a role of epigenetic modifications in this process.

Figure 6: The majority of DEGs are associated with DMRs between mature tolerogenic and inflammatory DCs. ***(A)*** *Pie chart representing all DEGs, categorized depending on the genomic location of DMCs associated with that DEG. The promotor region was identified as being 0–2.5 kb upstream of the TSS. When there was no associated DMC with that DEG, it was categorized as "remaining."* ***(B)*** *Methylation of the CD86 gene. Average gene expression of CD86 was obtained using the scaled coverage of the 11 samples in each group. Average DNA methylation level at each CpG on each group was calculated based on normalized DNA methylation level. Two DMLs (cg09644952 and cg01436254) were noted in the proximal promotor of CD86 (yellow highlighted area), and expression was lower in tolDCs compared with inflammatory DCs (log2FC = –0.49, Q = 0.009).* ***(C)*** *Similar methods were used for gene expression and DNA methylation of CD52. Although a significant difference in gene expression of CD52 was noted between tolerogenic and inflammatory DCs (log2FC = 4.48, Q < 0.0001), no DMLs near the gene body or in the promotor were observed. imDC, immature DC; imtolDC, immature tolDC; mtolDC, mature tolDC.*

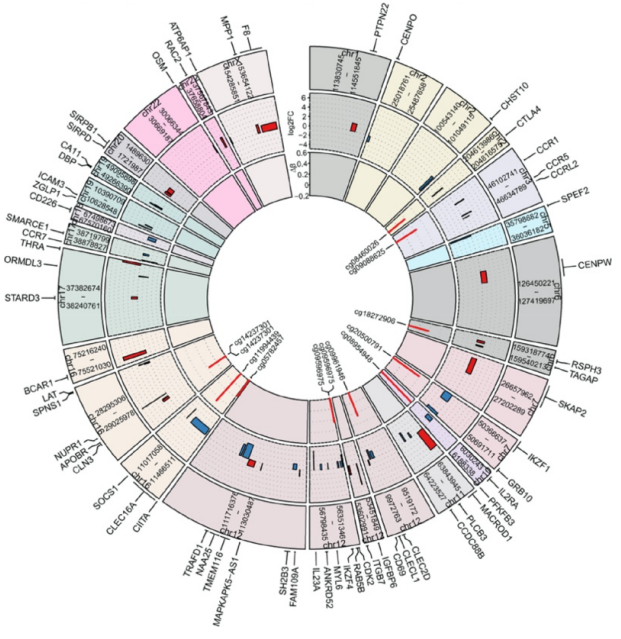


Figure 7: Multiple T1D risk genes were differentially expressed and associated with DMLs between tolerogenic and inflammatory DCs. *Outer layer of graph shows the genomic locations of 62 differentially expressed T1D risk genes between mtolDCs (n = 11) and inflammatory mDCs (n = 11) by name in a circos plot. Middle layer of graph shows the gene expression difference in log2FC between tolDCs and mDCs visualized by box plots, where the x-axis represents the genomic locations of the genes (from TSS to end site) in the corresponding T1D-suspectible regions and the y-axis represents log2FC. Upregulated expression in tolDCs versus mDCs is depicted in red and downregulated expression in blue. Inner layer of graph shows that among the 62 genes, 12 contained DMCs at their gene bodies and/or 5-kb flanking regions (5 kb upstream of TSS [promoter] and 5 kb downstream of gene bodies). The methylation level difference (Δβ, mtolDCs versus mDCs) of the most significant DMCs (IDs shown inside the plot) for each of these 12 genes is plotted in the inner layer of the graph, with red representing hypermethylation and blue representing hypomethylation. IDs, identifiers; mtolDCs, mature tolDCs.*

Discussion

Ensuring reproducible and stable cellular products is crucial for the implementation of a cellular therapy, as the protocol should deliver stable and similar cell products regardless of processing site or health status. Other cellular therapies, such as mesenchymal stromal cells, have been under scrutiny lately, as reproducibility between centers and even donors within the same center has been poor (42). In the authors' study, it was found that tolDCs were reproducible between centers and between healthy subjects and T1D patients. In addition, tolDCs seemed more homogeneous in phenotype and function than inflammatory DCs. The variability among inflammatory DCs supports the authors' experience that using these as a reference for the release of tolDCs for clinical use can be troublesome. In an effort to standardize the production of tolDCs and improve reproducibility, minimum information regarding tolerogenic antigen-presenting cells was introduced in recent years (43). The next step would be to standardize tolDC production on the basis of clinical therapeutic efficacy, which is currently still lacking. In this respect, the authors are presently developing independent and stable release criteria for tolDCs, which might include CD86 and CD52 (27), which the current study has now shown to be epigenetically regulated in tolDCs.

The effect of T1D on the production of tolDCs was also studied at the transcriptome and methylome levels. At transcription level, only minimal differences between tolDCs from T1D patients and healthy controls were noted. Overall, only four DEGs were found in the mature tolDCs of T1D donors compared with healthy controls, and none were related to immunological function. Recently, a report warned about the negative impact of hyperglycemia in T1D patients on tolDC function (44,45). It should be noted, however, that all D-Sense T1D patients had tight blood glucose control since hemoglobin A1c > 64 mmol/mol (8%) was an exclusion criterion of this trial (27). Several other groups have reported that monocytes and DCs of T1D patients are different from healthy subjects, but these studies used protocols different from that used by the authors (46-49). Although monocyte frequencies were similar between T1D patients and healthy subjects, monocytes from T1D patients had alterations in their endoplasmic reticulum and oxidative stress pathways at the RNA level (49,50). Furthermore, the authors have reported previously that monoyctes of T1D patients have 155 hypomethylated and 247 hypermethylated regions at the DNA methylation level compared with healthy subjects (49,51,52). The authors' current data, however, match experiences from investigators studying tolDCs for rheumatoid arthritis or multiple sclerosis, reporting no effect of health status on tolDC production (53,54).

Modulation by VD3 overrides clinical phenotypes, which is especially relevant in the context of T1D, where VD3 supplementation in early childhood reduces the risk of developing T1D later in life (55,56). The protective effect of VD3 on T1D development

could be due to the binding of the vitamin D receptor to autoimmune risk genes (57). Indeed, VD3 changed the expression of half of the risk genes associated with multiple sclerosis in mouse T cells (58). In accordance with the authors' previous study (17), one third of candidate T1D risk genes were differentially expressed, implying that VD3 supplementation may override genetic risk predisposition for T1D. Furthermore, the authors found in this study that up to 80% of these genes contain DMCs between tolDCs and mDCs in the promoter, gene body or nearby region. This is important, as epigenetics have been reported to influence the expression of T1D risk genes in the monocytes of T1D patients compared with healthy controls (59,60). VD3 may reduce this disparity in T1D patients, and the present results support the notion that VD3 supplementation early in life has a longstanding protective effect, as DNA methylation is thought to be a stable marker (12,55).

The stability of tolDCs was validated in two stages. First, mature tolDCs resisted perturbation with inflammatory stimuli, in line with what has been observed for tolDCs in rheumatoid arthritis (53). Out of all phenotypic, functional and metabolic markers tested, only CD209 decreased in both tolerogenic and inflammatory DCs after additional inflammatory stimuli. With CD209 also downregulated upon anti-inflammatory treatment (i.e., dexamethasone) (61), low CD209 could be associated with an anti-inflammatory phenotype. These results are reassuring, as concerns have been raised about the possibility of an *in vivo* conversion of tolDCs to a pro-inflammatory phenotype (62).

Second, epigenetic studies revealed that several thousand DMLs found between tolerogenic and inflammatory DCs were mostly already present at the immature state. *IL1RN* is one of the few genes consistently upregulated in tolDCs across several previous studies as well as the authors' present study (63,64). In addition, the authors showed that *IL1RN* contained several DMCs in its promotor region, which may explain the consistent expression of *IL1RN* across studies. IPA of genes associated with both differential expression and methylation revealed enrichment of NFκB, TNF, IFN-γ, CD40L and IL-13, suggesting a stable, epigenetically controlled regulation of these important inflammatory pathways. TNF signaling proved to be crucial in inducing regulatory T cells from naive CD4 T cells by tolDCs, and CCL24 attracts naive CD4 T cells (21). *CCL24* was upregulated, whereas *CCL13*, which is associated with chronic inflammatory diseases, was downregulated in tolDCs versus inflammatory DCs (65,66). Both chemokines were differentially methylated in their promotor regions, pointing to the induction of a stable anti-inflammatory environment by tolDCs.

The authors' data also support the observation that maturation of immune-activating DCs results in widespread DNA demethylation (67). Instead, matured tolDCs mostly retained the DNA methylation status of their immature phase, which is in accordance with the

hypothesis that tolDCs are "locked" in an immature state. The transcription factors *KLF9* and *JUNB* were similarly upregulated in immature DCs and mature tolDCs, as opposed to mature inflammatory DCs (63). Furthermore, the enrichment of *KLF9* and *JUNB* binding sites at DMRs between tolDCs and inflammatory DCs suggests potential important roles of these transcription factors in gene expression regulation of tolDCs via DNA methylation.

The region that was differentially demethylated in inflammatory DCs compared with tolDCs was associated with lymphocyte differentiation and activation. This finding corroborates the higher T-cell stimulatory capacity of inflammatory DCs compared with tolDCs that is consistently found at the RNA, protein and functional level. Furthermore, the DNA methylation levels at these same CpGs are comparable in immature inflammatory DCs and tolDCs (both before and after maturation), pointing toward a common functional asset of reduced T-cell activation by these cell types. Other characteristics are diverging, however. The authors show, namely, that tolDCs do not merely retain their immature state but have an independent resetting of their phenotype, as has been recently proposed (63). For instance, morphology is an easily detectable and distinguishable feature separating tolDCs from inflammatory DCs. Within 3 days of culture, tolDCs can be separated from inflammatory DCs by visual means on the basis of their spindle shape and plate adherence, which persists throughout the 8 days of culture (20). Indeed, DNA methylation could play an important role in cell morphology, as the most significant enriched biological function in genes containing DMCs in mature tolDCs versus inflammatory DCs proved related to cell morphology. By contrast, differences in immunological pathways dominate at the transcriptome level (17). Interestingly, these pathways were enriched in genes containing DMCs upon maturation of inflammatory DCs, suggesting the involvement of DNA methylation in these pathways as well. This points to the divergence of tolDCs from inflammatory DCs in terms of cell shape and cellular movement, whereas they retain the hypoimmunogenic features of immature DCs. Some caution is warranted when interpreting the role of DNA methylation in gene expression, however, as gene expression can precede DNA methylation in response to activation of monocyte-derived DCs (68).

Conclusions

VD3 plus dexamethasone induced epigenetic modifications at key loci in tolDCs, which may contribute to the observed stability of tolDCs in phenotype and function. Furthermore, reproducibility was shown with regard to phenotypic, transcriptomic and methylomic tolDC profiles regardless of manufacturing center or health status. Together, this reinforces the feasibility of and attraction for the implementation of tolDCs as a stable, reproducible immunomodulatory therapeutic strategy in T1D and other autoimmune disorders.

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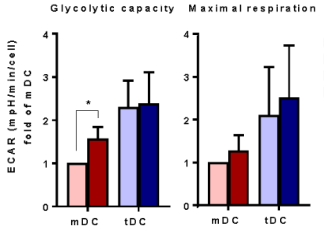
Supplementary Information

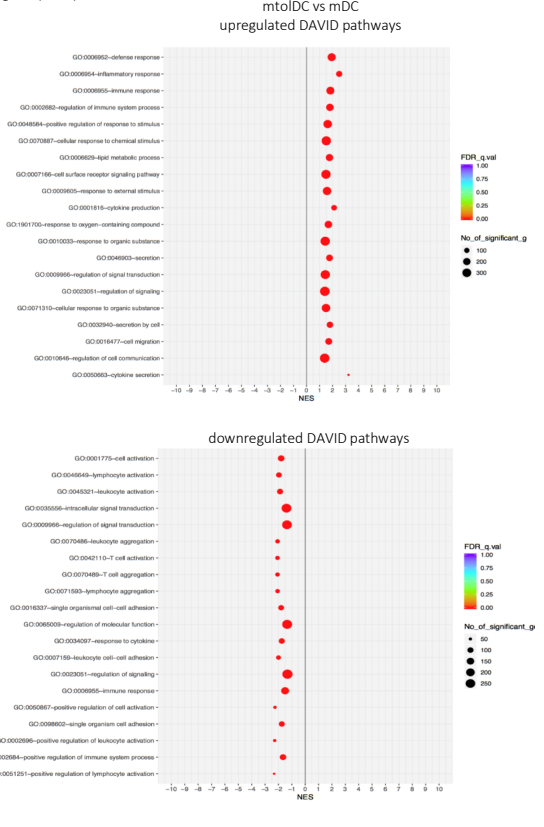


Supplementary Figure 1: Analysis of the metabolic activity of tolerogenic and inflammatory dendritic cells treated with CD40L. *Tolerogenic (tDC) and inflammatory DCs (mDC) were stimulated with CD40L for 18 hrs (dark shade bars) or left unstimulated (light shade bars), and consequently their cell metabolism was measured by Seahorse (n=3). Glycolytic capacity and maximal respiration was calculated from the extracellular acidification rate (ECAR) and from the oxygen consumption rate (OCR), respectively. Unstimulated tolDC showed higher glycolytic capacity and maximal respiration compared to unstimulated mDC. There was a significant increase in glycolytic capacity noted in the mDC group after CD40 ligation (unpaired student's t test; p=0.0187), whereas no significant difference was noted in the tolerogenic DC.*

Supplementary Figures 2A-D (on the following pages): Bubble charts of pathway analyses of transcriptomic data of different cell type comparisons. *For every cell type comparison, the most significant GSEA and KEGG pathway analyses results are shown (n=11 vs n=11; for every cell type comparison). A color gradient describes the q values with red being most significant (q= 0.00) and blue being least significant (q=1.00). The size of the bubbles describes the number of significant genes within the pathway ranging from 50 to 200 genes. (A) mtolDC vs mDC (B) imtolDC vs imDC (C) mtolDC vs imtolDC (D) mDC vs imDC.*

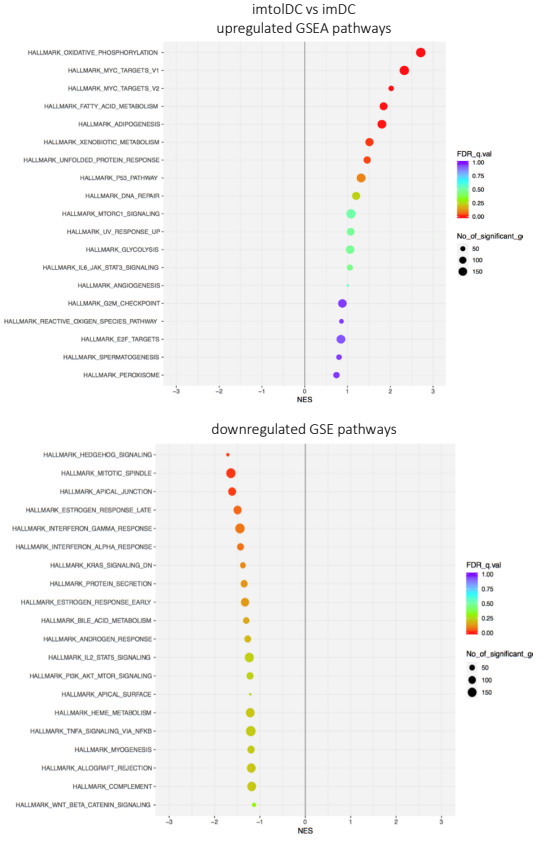
Supp. Fig. 2A

Supp. Fig. 2A (cont.)



Supp. Fig. 2A (cont.)

Supp. Fig. 2B



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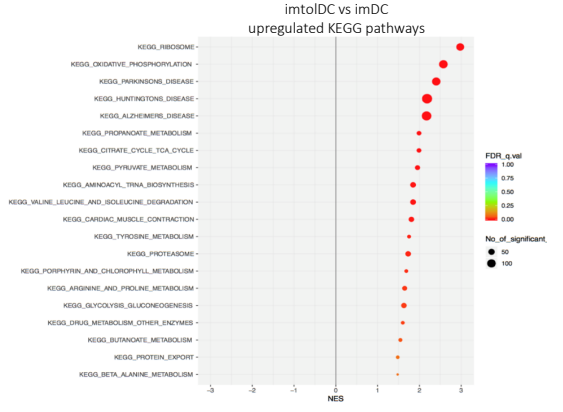

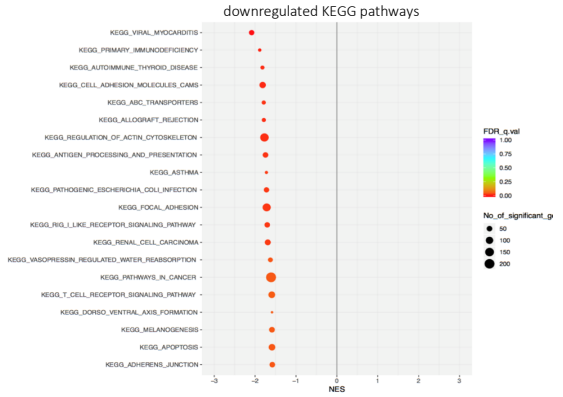

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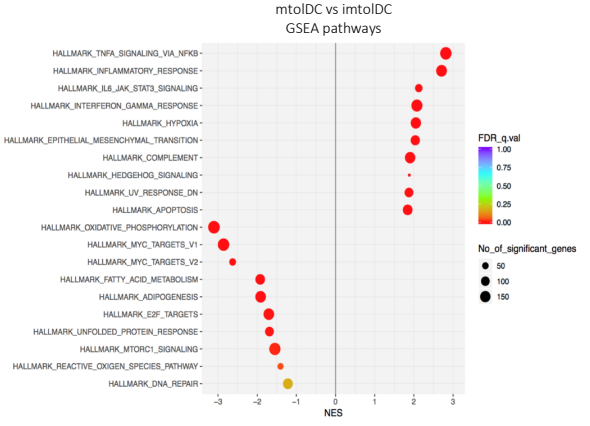

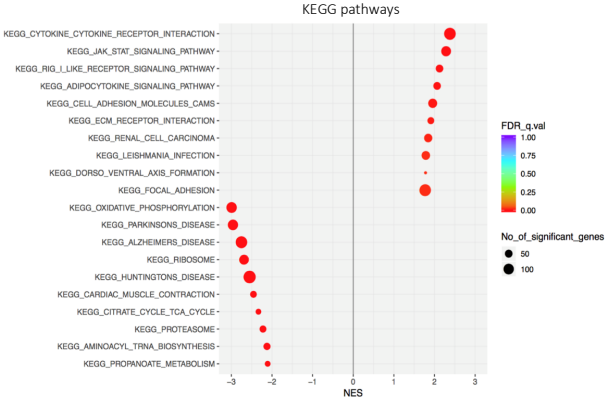

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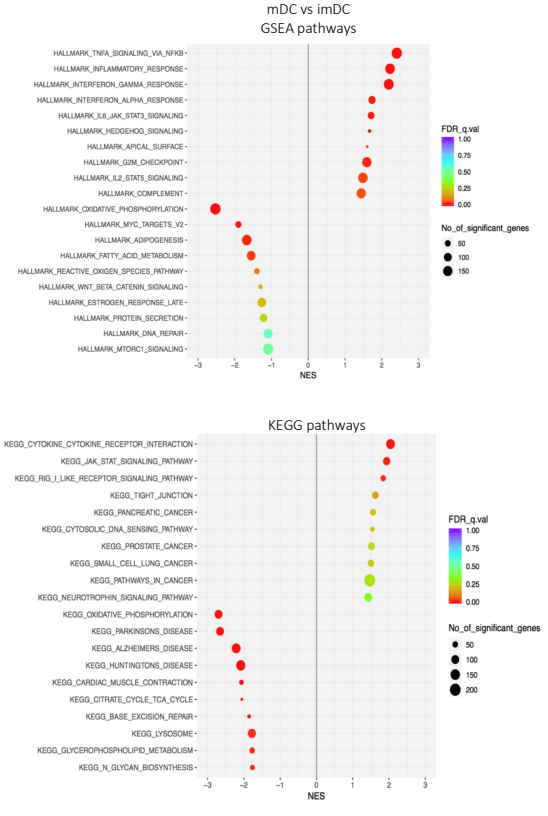
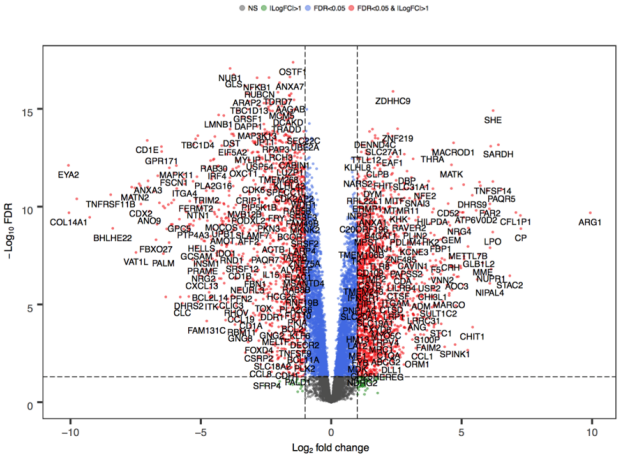
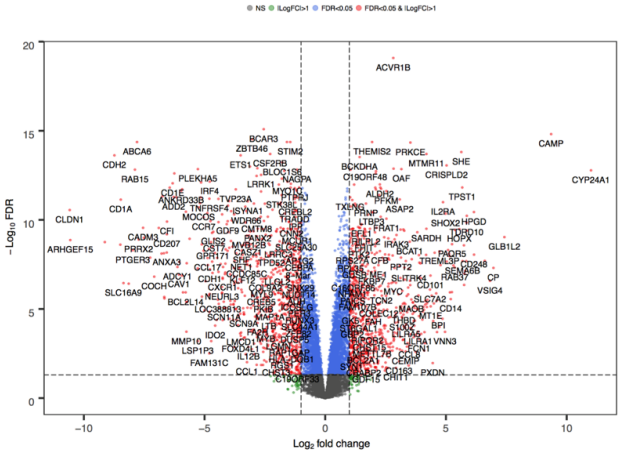
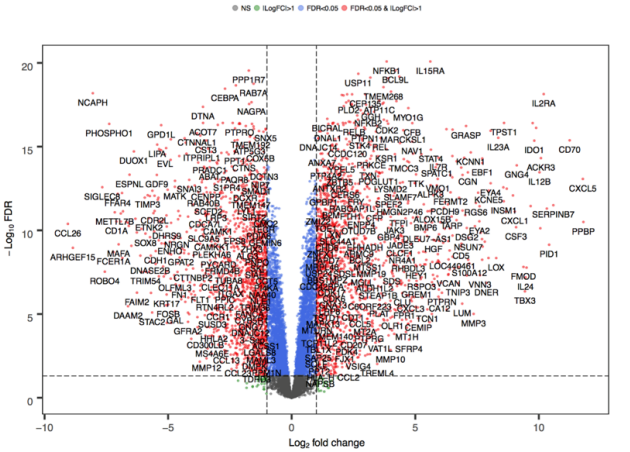




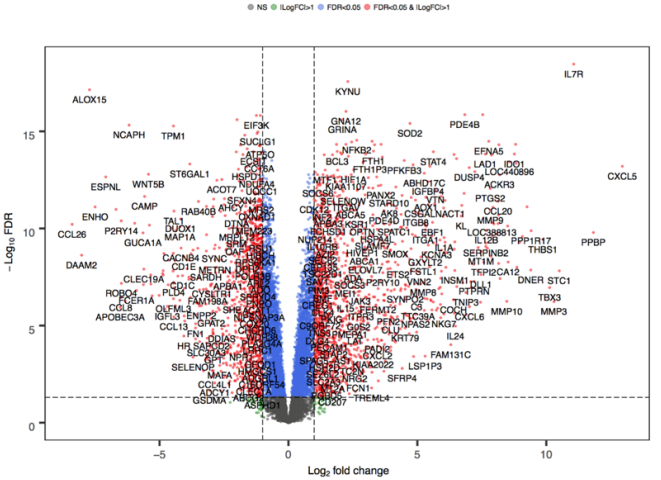
Supplementary Figure 3: Volcanoplot of differentially expressed genes between mature tolerogenic and inflammatory DCs. *Volcanoplot represent differentially expressed genes (DEGs) between mature tolerogenic (n=11) and inflammatory (n=11) dendritic cells. Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



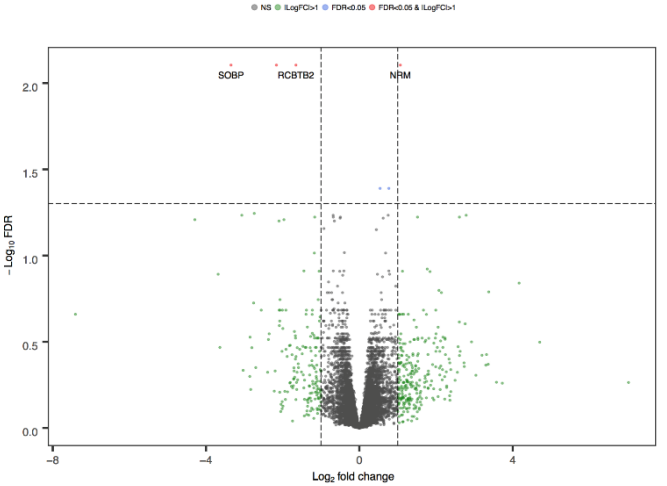
Supplementary Figure 4: Volcanoplot of differentially expressed genes between immature tolerogenic and inflammatory DCs. *Volcanoplot represent differentially expressed genes (DEGs) between immature tolerogenic (n=11) and immature inflammatory (n=11) dendritic cells. Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



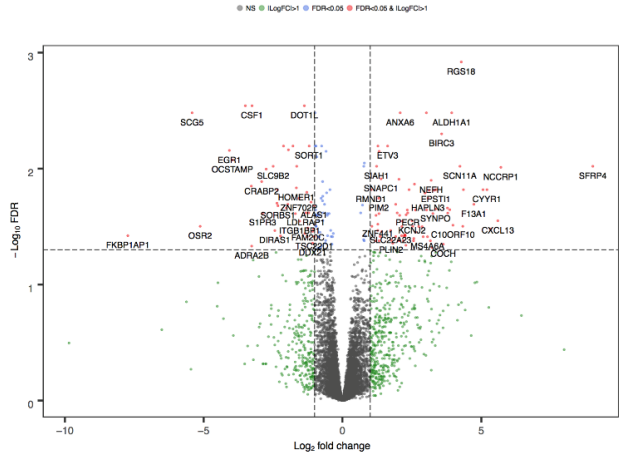
Supplementary Figure 5: Volcanoplot of differentially expressed genes between mature and immature inflammatory DCs. *Volcanoplot represent differentially expressed genes (DEGs) between mature (n=11) and immature (n=11) inflammatory dendritic cells. Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



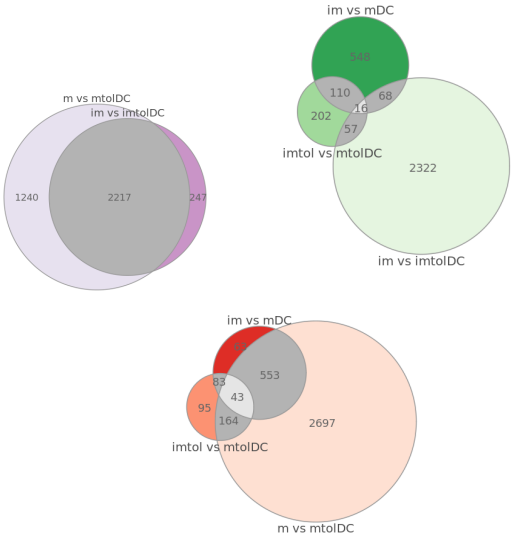
Supplementary Figure 6: Volcano plot of differentially expressed genes between mature and immature tolerogenic DCs. *Volcanoplot represent differentially expressed genes (DEGs) between mature (n=11) and immature (n=11) tolerogenic dendritic cells. Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



Supplementary Figure 7: Volcanoplot of differentially expressed genes between mature tolerogenic DCs produced from healthy compared to T1D donors. *Volcanoplot represent differentially expressed genes (DEGs) between mature tolerogenic DCs from healthy (n=3) compared to T1D (n=5) donors. All cells were produced in Leiden University Medical Center. Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



Supplementary Figure 8: Volcanoplot of differentially expressed genes between tolerogenic DCs produced in Leiden University Medical Center compared to City of Hope. *Volcanoplot represent differentially expressed genes (DEGs) between mature tolerogenic DCs produced in Leiden University Medical Center (n=3) compared to City of Hope (n=3). Red symbols represent DEGs with a significance of <0.05 FDR with a log fold change (FC) >1. Blue symbols represent genes differentially expressed with a FDR <0.05, but with a log FC <1, whereas green symbols represent genes expressed with a log FC >1, but a FDR >0.05. All red symbols are accompanied by the corresponding gene name of that DEG.*



Supplementary Figure 9: Venn diagrams of overlapping differentially methylated CpGs (DMC) in different cell type comparisons. *The number of DMCs is written in the circle and the number of overlapping DMCs are depicted in a gray circle. m (and mDC) = mature inflammatory dendritic cell; mtolDC= mature tolerogenic dendritic cell; imtol (and imtolDC) = immature tolerogenic dendritic cell; im= immature inflammatory dendritic cell.*

Genes	logFC	PValue	FDR	Indicator
CD14	1.390213	0.061641	0.083986	
CD52	4.483334	4.22E-12	6.99E-11	UP
CCR1	2.261248	6.98E-05	0.000166	UP
CCR5	2.279056	2.11E-05	5.56E-05	UP
FCGR3A	2.394821	0.000751	0.001471	UP
CD163	2.02353	0.041889	0.058969	
MRC1	1.916532	0.000309	0.00065	UP

Supplementary Table 1: Expression of immature dendritic cell markers in mtolDC versus mDC *Table shows differential expression of genes between mature tolerogenic (n=11) and inflammatory dendritic cells (n=11) in log fold change (FC), p-value, and false discovery rate (FDR). The final column indicates whether the gene was identified as a differentially expressed gene according to a FDR <0.05 and a logFC>1 (UP) or not (blank).*

Comparisons	Baseline	Compared to	No. of DMCs
mtolDC vs mDC	M	Mtol	3457
imtolDC vs imDC	im	imtol	2463
mDC vs imDC	im	m	742
mtolDC vs imtolDC	imtol	mtol	384
T1D vs healthy_mtol	LUMC mtol	D-sense mtol	26
T1D vs healthy _imtol	LUMC imtol	D-sense imtol	7
T1D vs healthy _m	LUMC m	D-sense m	6
T1D vs healthy _im	LUMC im	D-sense im	9
Location effect_mtol	COH mtol	LUMC mtol	13
Location effect _imtol	COH imtol	LUMC imtol	12
Location effect _m	COH m	LUMC m	11
Location effect _im	COH im	LUMC im	10

Supplementary Table 2: Number of differentially methylated CpGs (DMC) in different cell type, health status, and location comparisons *Table indicates the number of DMCs (column 4) between different comparisons (column 1). Column 2 indicates the baseline of the comparison and column 3 the cell type the comparison is made with: m indicates mature inflammatory DC; mtol mature tolerogenic DC; im immature inflammatory DC; imtol immature tolerogenic DC; LUMC Leiden University Medical Center; D-sense D-sense clinical trial in LUMC; COH City of Hope.*

DMCs were selected by the following selection criteria:

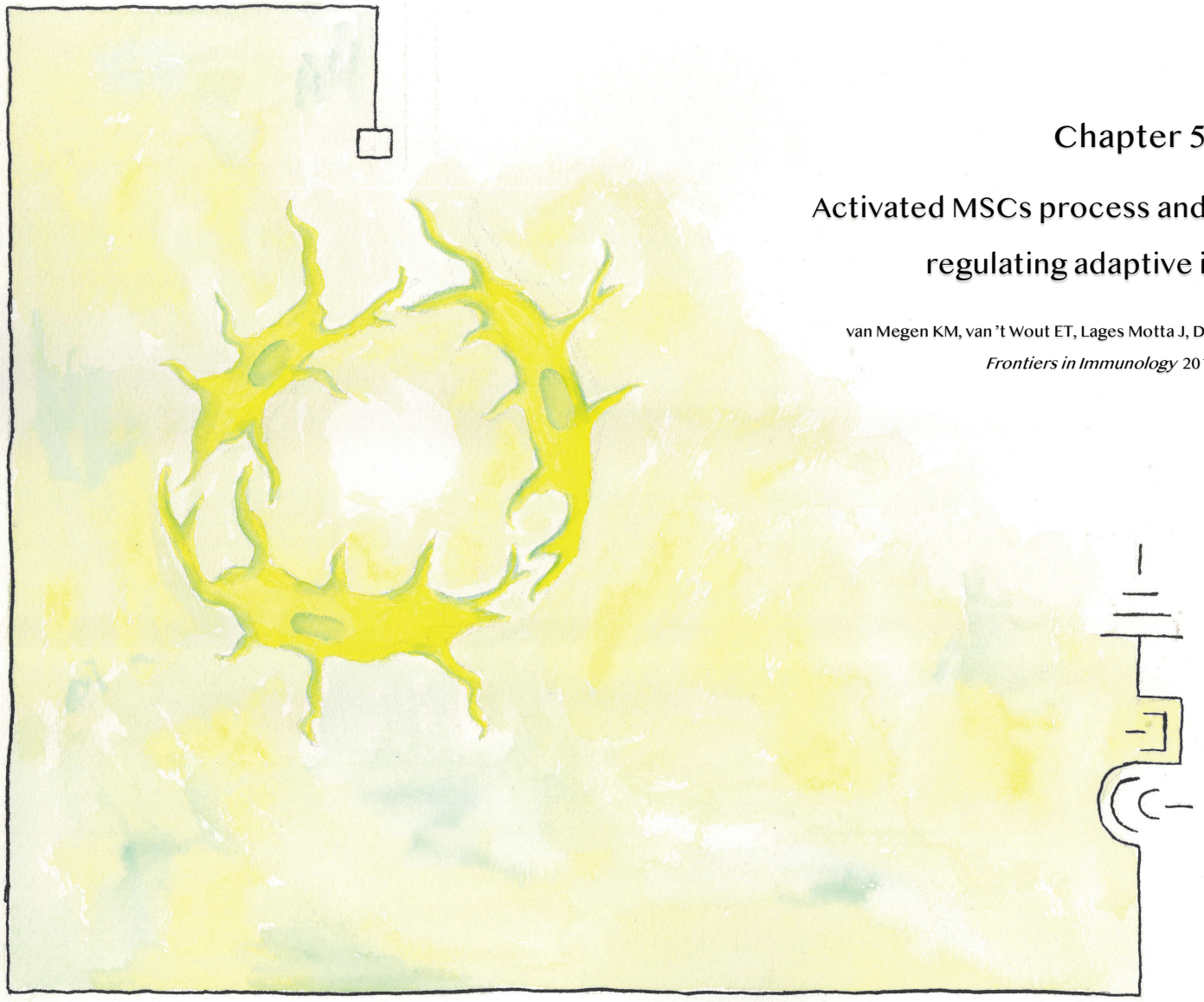
1. Down more than 0.15 when inflammatory DCs mature;
2. Changes less than 0.15 when tolerogenic DCs mature;
3. The difference between above two > 0.15 (inflammatory DCs changes more).

Term	PValue	Genes	FDR
lymphocyte differentiation	8.6E-7	KLF6, TNFSF4, IL2RA, IL23R, IKZF1, BRAF, FLT3, RELB, PRKDC, ITGA4, IL7R, TESPA1, DCLRE1C, DOCK2, CD86, CCR6	0.0015
leukocyte differentiation	9.3E-6	KLF6, IL23R, TNFSF4, IL2RA, IKZF1, BRAF, FLT3, RELB, PRKDC, ITGA4, IL7R, TESPA1, DCLRE1C, DOCK2, CD86, CCR6, GPR55, RUNX1	0.0168
leukocyte cell-cell adhesion	1.4E-5	TNFSF4, IL2RA, IL23R, BRAF, RELB, PRKDC, IDO1, ITGA4, CCL5, IL7R, TESPA1, DOCK2, CD86, CCR6, CD44, CD274, PPP3CA, TNIP1	0.0266
lymphocyte activation	4.0E-5	KLF6, IL23R, TNFSF4, IL2RA, IKZF1, BRAF, FLT3, RELB, PRKDC, IDO1, ITGA4, IL7R, CCL5, TESPA1, DCLRE1C, DOCK2, CD86, CCR6, CD274, PPP3CA	0.0742
regulation of activated T cell proliferation	4.9E-5	CD86, IL23R, IL2RA, TNFSF4, CD274, IDO1	0.0896

Supplementary Table 3: Top DAVID analyses results of genes associated with differentially methylated CpGs (DMCs) in red box of DMC heatmap. *Table shows top results of DAVID analysis on DMCs identified in the red box in Figure 5C of mature tolerogenic dendritic cells (n=11) compared to mature inflammatory dendritic cells (n=11). FDR= false discovery rate.*

Term	PValue	Genes	FDR %
regulation of cell shape	5.52E-04	CCL24, GNA13, CCL13, FYN, BAIAP2, HEXB, CFDP1	0.86
positive regulation of actin filament polymerization	3.14E-04	CCL24, MYO1C, CARMIL1, BAIAP2, MLST8	0.49
heart development	2.20E-03	GNAQ, RPS6KA2, HOPX, AKAP13, PRKDC, CXADR, TAB2	3.39
positive regulation of GTPase activity	2.74E-03	CCL24, CCL13, A2M, RASGRP3, GNAQ, FYN, TBCD, ASAP2, RIN2, FGF23, ARHGAP45, AKAP13	4.21

Supplementary Table 4: DAVID analysis of biological process pathways on all hyper- and hypomethylated regions comparing mature tolerogenic with inflammatory dendritic cells. *Table shows top results of DAVID analysis on differentially methylated regions of mature tolerogenic dendritic cells (n=11) compared to mature inflammatory dendritic cells (n=11). FDR= false discovery rate.*



Chapter 5

Activated MSCs process and present antigens regulating adaptive immunity

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Abstract

Mesenchymal stromal cells (MSCs) are inherently immunomodulatory through production of inhibiting soluble factors and expression of immunosuppressive cell surface markers. We tested whether activated MSCs qualify for the induction of antigen-specific immune regulation. Bone marrow derived human MSCs were activated by interferon-γ and analyzed for antigen uptake and processing and immune regulatory features including phenotype, immunosuppressive capacity, and metabolic activity. To assess whether activated MSC can modulate adaptive immunity, MSCs were pulsed with islet auto-antigen (GAD65) peptide to stimulate GAD65-specific T-cells. We confirm that inflammatory activation of MSCs increased HLA class II, PD-L1, and intracellular IDO expression, whereas co-stimulatory molecules including CD86 remained absent. MSCs remained locked in their metabolic phenotype, as activation did not alter glycolytic function or mitochondrial respiration. MSCs were able to uptake and process protein. Activated HLA-DR3-expressing MSCs pulsed with GAD65 peptide inhibited proliferation of HLA-DR3-restricted GAD65-specific T-cells, while this HLA class II expression did not induce cellular alloreactivity. Conditioning of antigen-specific T-cells by activated and antigen-pulsed MSCs prevented T-cells to proliferate upon subsequent activation by dendritic cells, even after removal of the MSCs. In sum, activation of MSCs with inflammatory stimuli turns these cells into suppressive cells capable of mediating adaptive regulation of proinflammatory pathogenic T-cells.

Introduction

Mesenchymal stromal cells (MSCs) are non-hematopoietic cells that can easily be sourced from various tissues, including bone marrow (1). They have been widely used clinically to improve the outcome of hematopoietic stem cell and solid organ transplants and to treat graft-vs. -host disease (2, 3). Consequently, safety has been established in terms of toxicity and tumerogenicity (3). The immunomodulative properties of MSCs also make these excellent candidates for cellular therapies targeting inflammatory and autoimmune disorders, including type 1 diabetes (T1D) (3). T1D is a T-cell mediated autoimmune disease in which autoreactive T-cells selectively kill insulin-producing beta-cells in the pancreas (4). Interestingly, MSCs have also been investigated for their potential to regenerate beta-cells, or to contribute to regeneration of beta-cells, which is another strategy to counter T1D (5, 6).

The hypoimmunogenic nature of MSCs could be responsible for evading alloreactivity as by definition they lack HLA class II (7). Hence, the use of allogeneic MSCs as a cellular therapy appears attractive as it is safe and enables "off-the-shelf" therapeutics (3). Immunomodulation by MSCs may be achieved by a range of soluble factors including indoleamine 2,3-dioxygenase (IDO) (1). In addition, cell-cell contact involving programmed death-ligand 1 (PD-L1) resulted in inhibition of T-cell proliferation and induction of T regulatory cells (2, 8).

Pro-inflammatory cytokines such as interferon gamma (IFN-γ) induce HLA class II expression on MSCs (9), which endorses antigen-presenting capacity of MSCs to CD4 T-cells, but could also affect their hypoimmunogenic nature. Indeed, mouse and human MSCs can act as unconventional antigen presenting cells, stimulating proliferation of T-cells (10–12, 14). Therefore, concerns have been raised about the potential to increase the immunogenicity of MSCs by activating them, but this has not consistently been substantiated (2). While cellular and humoral alloreactivity against MHC-mismatched MSCs have been reported in animal models, human MSCs did not show alloreactivity *in vitro* (13, 15). Indeed, activation of human MSCs enhanced their ability to inhibit allogeneic T-cell proliferation and reduced pro-inflammatory cytokine production in co-cultures (16–18).

Activation of MSCs may enable their use as an antigen-specific therapy, which is the long-sought objective in immunotherapy (19). While non-specific immunotherapies seem insufficient to intervene in auto-immune diseases and cancer (20), antigen-specific therapies using either antigenic peptide alone (21) or with cellular adjuvants such as antigen-pulsed dendritic cells (22, 23), or with CAR-T-cells (24), have emerged with promising outcomes. MSCs, too, have been tested as cell therapy to modulate adaptive immunity non-specifically (25–29). MSCs or their microvesicles inhibited an inflammatory

response against diabetogenic peptides in patients with T1D and non-obese diabetic (NOD) mice (25, 26).

In the first clinical trial treating T1D patients, non-activated autologous MSCs preserved or even increased c-peptide response to a mixed meal tolerance test (MMTT) (30). This illustrates that their mere immunomodulatory nature may already affect the course of the disease favorably. Turning MSCs into antigen-specific adjuvants would increase the appeal to engage MSCs as a cellular therapy. This study set out to determine whether peptide-pulsed human MSCs can inhibit antigen-specific responses *in vitro* as a critical step to clinical translation of MSCs as an adaptive, antigen-specific immunotherapy in autoimmunity.

Materials and Methods

Human MSC Culture, Activation, and Antigen Processing

Bone marrow derived human MSCs were obtained from healthy individuals as described previously (31). Briefly, bone-marrow was collected from patients undergoing hip or knee replacement surgery at the Leiden University Medical Center (LUMC). Mononuclear cells were isolated by gradient centrifugation and cultured in "MSC medium" consisting of Dulbecco's Modified Eagle's (DMEM) low glucose medium (Life Technologies, New York, USA) with 10% Fetal Bovine Serum (FBS) (Sigma-Greiner, Wemmel, Belgium) and 100 IU/ml Penicillin and 100 IU/ml Streptomycin (Life Technologies). Next day, non-adherent cells were removed and cells were grown to confluence. Cells were harvested at ~90% confluency by trypsinizing the cells for 9 min at 37°C with 0.05% trypsin-EDTA (Life Technologies). The MSCs used for the current study have been characterized by flow cytometry and lineage differentiation in accordance with the minimal criteria for defining MSCs and used for clinical trials (32). In between passages cells could be cryopreserved in liquid nitrogen in 50% MSC medium, 40% FBS, and 10% Dimethyl Sulfoxide (DMSO). MSCs were collected and stored between passage 3 and 7.

Where applicable, MSCs were activated with 1,000 IU/ml IFN-γ (MSC-γ) (R&D systems) or by culturing MSCs in twice diluted supernatant of an autoimmune T-cell clone (PM1#11) isolated from a prediabetic patient and reactive to islet antigen glutamic acid decarboxylase 65 (GAD65) for 48 h (33). For antigen uptake and presentation, cells were incubated with labeled Ovalbumin (OVA-DQ, Invitrogen) that becomes fluorescent once it has been taken up and proteolytically degraded in the cell. 1×10^4 MSCs were incubated with 5 µg OVA-DQ for 4 h at 37 or 4°C for control of spontaneous uptake/processing, and analyzed by flow cytometry and fluorescence microscopy (Xcyto-10). For microscopy, cells were visualized with Blue Mask (diluted 1:1,000 in PBS) upon 30 min incubation at room temperature.

Human Monocyte Derived Dendritic Cells and T Cells

Monocyte-derived dendritic cells (DC) were generated as described previously (34). In short, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of HLA typed healthy human donors (Sanquin, Amsterdam, The Netherlands) by density gradient centrifugation. Monocytes were selected by positive selection using CD14-specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in RPMI-1640 (Life Technologies) supplemented with 8% fetal bovine serum (heat-inactivated FBS, Sigma F0804), 100 IU/mL Penicillin and 100 IU/mL Streptomycin (Pen/Strep, Life Technologies), 2 mM L-glutamin (Glut, Life Technologies), 500 IU/mL recombinant IL-4 (Invitrogen, Breda, Netherlands) and 800 IU/mL recombinant GM-CSF (Invitrogen) for 6 days to obtain immature DC (iDC). iDC were matured in a 2-day culture using 100 ng/mL lipopolysaccharide (LPS: Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Dendritic cells used in all experiments were HLA-matched to the PM1#11 clone (HLA-DR3). CD14 negative cells were preserved in liquid nitrogen and used in different assays as Peripheral Blood Lymphocytes (PBLs). The PM1#11 clone was derived from a prediabetic patient after informed consent. The clone is HLA-DR3 restricted and specific for $GAD65_{339–352}$ (33). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Lonza) supplemented with 10% pooled human serum, Pen/Strep, and glutamine (Glut).

Cytokine Assays

Supernatants from activated and non-activated MSCs and GAD-specific T-cell clones were harvested and analyzed for cytokine analysis with a Luminex kit (Bio-Rad; Hercules, CA) according to the manufacturer's protocol.

Flow Cytometry

MSCs were stained with 1:5,000 Live/Dead Fixable Blue Dead Cell Stain Kit (Life Technologies) for 20min according to manufacturer's protocol, after which cells were incubated with a panel of monoclonal antibodies (**Suppl. Table 1**) for 30min on ice. Cells were washed in FACS buffer containing 1% FBS and 0.05% Sodium Azide (Sigma-Aldrich) and analyzed using FACS Canto and Fortessa (BD). Data was analyzed using FACS DIVA v8 (BD Biosciences) and FlowJo v10 software (Ashland, Oregon, USA). The gating strategy is presented in the supplement (**Suppl. Figure 1**).

Real-Time Metabolic Characterization

The XF^e96 extracellular flux analyzer (Seahorse Bioscience, North Billerica, USA) was used to measure mitochondrial oxygen consumption rate (OCR, O_2 mpH/min) and extracellular acidification rate (ECAR, mpH/min). Prior to experiments, optimization with regards to cell number and concentration of compounds was performed. Subsequently, MSCs were harvested, counted, and plated (1×10^4 cells/well) in MSC medium supplemented or not

with 1,000 IU/mL IFN-γ and incubated for 48 h at 37°C. On the day of analysis, MSCs were thoroughly washed (3x) in either glycolysis stress test assay medium (DMEM base, 2 mM L-glutamine; pH 7.35) or mitochondrial stress test medium (DMEM base, 2 mM L-glutamine, 1 mM pyruvate, 25 mM glucose; pH 7.35) and incubated in a non-CO_2 incubator at 37°C for 1h. For the glycolysis stress test the following compounds were used in the subsequent stages: basal (no drugs), glycolysis (10mM glucose), glycolytic capacity (1μM oligomycin), and glycolysis inhibition (50 mM 2-DG). For the mitochondrial stress test the following compounds were used in the subsequent stages: basal respiration (no drugs), ATP production inhibition (1μM oligomycin), maximal respiration (0.5 μM FCCP), and electron transport chain inhibition (0.5 μM rotenone and 0.5 μM antimycin A).

Alloresponse, Suppression, and Antigen-Specific Proliferation Assays

HLA-typed human PBLs or PM1#11 cells were labeled with CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) by staining 1×10^6 cells/mL in PBS with 0.5 μg/mL CFSE for 2 min at room temperature. Subsequently, 1×10^5 CFSE-labeled cells were plated in a 96-well plate and used for the following assays. For all assays, cells were harvested after 4 days of culture and stained with monoclonal antibodies against CD3, CD4, and CD8 and analyzed for proliferation by flow cytometry, unless otherwise described.

PBL cells were incubated with either HLA-mismatched MSCs, MSC-γ, or DC at a ratio 10:1 or CD3/CD28 beads (ratio 1:1) to measure alloresponse (Dynabeads Human T-Activator, Thermo Fisher). PBL cells were stimulated with CD3/CD28 beads alone (ratio 1:1) or in the presence of MSC or MSC-γ (ratio PBL:MSC 10:1) to test suppressive capacity of MSCs. MSC-γ or DCs prepulsed for 4 h with 5 μg/mL $GAD65_{339–352}$ peptide or GAD65 protein, after being washed three times, were incubated with GAD-specific T-cells (PM1#11) to assess antigen-specific proliferation.

HLA-DR3 (matched) and HLA-DR13 (mismatched) MSC-γ were prepulsed with different concentrations of the $GAD65_{339–352}$ peptide (0.2, 1, 5 μg/mL) for 4 h and thoroughly washed for the antigen-specific inhibition co-culture experiment. Next, HLA-DR3 DCs (HLA-matched), prepulsed with 1 μg $GAD65_{339–352}$ peptide, and GAD-specific T-cells were added to the culture in a DC:MSC:PM1#11 ratio of 1:1:5. After 3 days, $[^3H]$-thymidine (0.5 μCi/well) was added for 18h, after which incorporation was measured using a liquid scintillation counter. Data shown is the mean of triplicates with the standard error of the mean (SEM). This experiment was replicated with CFSE-labeled PM1#11 cells.

HLA-DR3 matched or -mismatched MSC-γ were loaded with GAD peptide and incubated with GAD-specific T-cells in a ratio 1:10 MSC:T cell for the preconditioning assay. After 24 h, T-cells were harvested leaving adherent MSCs intact, washed in PBS and subsequently

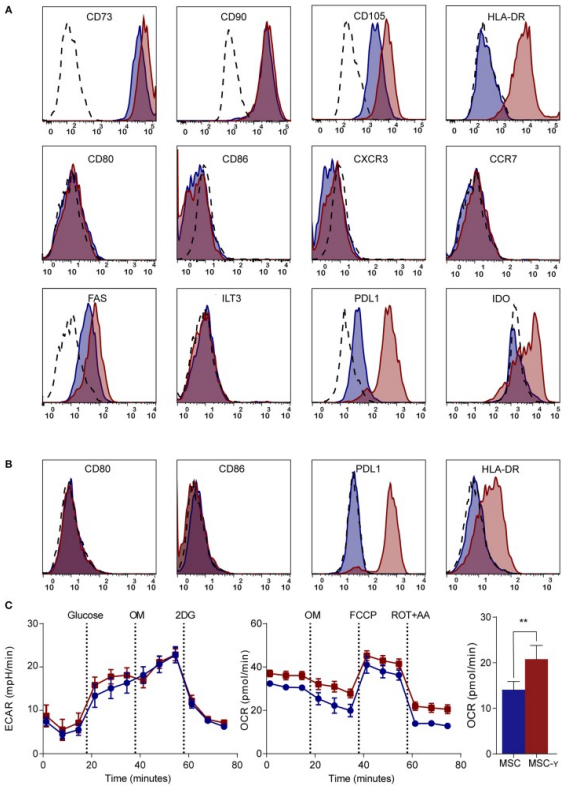

Figure 1: Phenotype and metabolism of activated MSCs. (A,B) *Flow cytometric analysis of the phenotype of non-activated (blue histograms) and activated (red histograms) MSCs and isotype controls (dashed histograms). In* ***(A)*** *activation is by IFN-γ and in* ***(B)*** *by supernatant of non-activated (blue) or activated (red) GAD-specific T-cells. The first row shows markers that identify MSCs; the second row represents co-stimulatory molecules and chemokine receptors; the third row identifies inhibitory markers. Representative histograms are shown (N =4).* ***(C)*** *Representative graphs of real-time metabolic data of non-activated (blue) and activated (red) MSCs as analyzed by the XF^e extracellular flux analyzer (Seahorse). In the glycolysis stress test (left graph) glucose is injected which induces glycolysis. Next, oligomycin (OM) is injected to induce maximal glycolytic capacity and 2-deoxy-D-glucose (2-DG) finally to inhibit glycolysis. In the mitochondrial stress test (right graph), basal respirationis measured, after which OM is added to inhibit ATP production. Consequently, carbonyl cyanide-4-phenylhydrazone (FCCP) is injected to induce maximal respiratory capacity. Lastly, rotenone and antimycin-A is added to block the electron transport chain. Only the non-mitochondrial respiration (bar graph) was significantly increased in activated MSCs (MSC-γ) compared to non-activated MSCs (MSC) (N = 4). An unpaired student's t-test was used to test statistical significance. ** p = 0.006. ECAR, Extracellular Acidification Rate; OCR, Oxygen Consumption Rate.*

the T-cells were cultured with DCs prepulsed with 1μg/mL GAD peptide (ratio 1:10 DC:T cell). A proliferation index (average number of divisions by dividing cells) was calculated by dividing the total number of divisions by the number of T-cells that proliferated.

Statistical Analysis

Data were analyzed for statistical significance using unpaired Student's *t*-test, one-way ANOVA or two-way ANOVA with, where appropriate, subsequent Tukey or Sidak's post-test for multiple comparisons using GraphPad Prism 7 (GraphPad Software, La Jolla, USA). In the figure legends is described which test is used. A $p < 0.05$ was considered significant.

Results

Activation of MSCs Increases HLA-DR Expression and Immune Inhibitory Markers, While Maintaining Their Metabolic Profile

MSCs generally lack HLA-DR expression, while this is needed for antigen presentation to CD4 T-cells (7). Activation of MSCs by IFN-γ increased the expression of HLA-DR without decreasing the expression of markers that characterize MSCs (CD73, CD90, and CD105) **(Figure 1A)**. Markers typically lacking on resting MSCs, namely CD34, CD45, CD14, and CD19 remained negative after activation (32) (**Suppl. Figure 2**). Besides these standard markers to characterize MSCs, activated MSCs were phenotyped more extensively by flow cytometry, analyzing expression of activating and inhibiting molecules involved in antigen-presentation and T-cell stimulation (35). Activation of MSCs with IFNg did not increase CD86 or CD80 expression. Similarly, chemokine receptors CCR7 and CXCR3, which are implicated in migration to lymph nodes and inflamed tissues (36), respectively, were not expressed before or after activation. Yet, activation of MSCs did enhance the expression of inhibitory molecule PD-L1, death receptor FAS and intracellular IDO expression, whereas inhibitory molecule ILT3 showed no change (**Figure 1A**). Next, we stimulated MSCs with the supernatant of activated autoreactive Th1-cells that we deem a more (patho)physiologically relevant stimulation when mimicking inflammatory insulitis than a single cytokine stimulation. The supernatant of the activated Th1-cells contained substantially higher levels of pro-inflammatory cytokines (IL-1β, IL-2, IL-5, IL-6, IL-8, IL-12, IL-13, IL-17, IFN-γ, and TNF-α), compared to the non-activated Th1-cell supernatant (**Suppl. Figure 3**). Similar to activation with IFN-γ alone, activating MSCs with activated

Th1-cell supernatant increased expression of HLA-DR and PD-L1 while keeping CD80 and CD86 expression low (**Figure 1B**). The supernatant of non-activated autoreactive Th1 did not activate MSCs in terms of surface marker expression. In addition to surface marker expression, we analyzed cytokine secretion by activated MSCs. Even after activation the secretion of pro- and anti-inflammatory cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α) was low and was not increased compared to non-activated MSCs (**Suppl. Figure 4**).

Since the metabolism of immune cells has proven pivotal in directing their immune activation or quiescence (37), a real-time metabolic characterization was performed to assess the effect of MSC activation on their metabolism. Activation did not impair the metabolism of MSCs, as indicated by unchanged mitochondrial respiration and glycolysis using a Seahorse analysis **(Figure 1C)**. Yet, non-mitochondrial respiration (measured by OCR) was increased in MSCs upon activation ($p = 0.006$; unpaired student's *t*-test) **(Figure 1C)**.

In summary, activation of MSCs enables their interaction with CD4 T-cells by upregulating HLA class II and selectively reinforces their inhibitory properties through increasing PD-L1 expression but not co-stimulatory molecules CD80 and CD86, while maintaining their resting metabolic profile.

Activated MSCs Do Not Stimulate Allo-Reactive T Cells but Enhance Immunosuppressive Capacity

The lack of HLA-DR expression promotes the immune privileged state of MSCs (1). This would imply that inducing HLA-DR expression on activated MSCs may cause concerns regarding increasing exposure to allo-reactive CD4 T-cells. To investigate this, non-activated or IFNγ-activated MSCs (MSC-γ) were cocultured with HLA-mismatched lymphocytes (PBL) and T-cell proliferation was measured using a CFSE-dilution assay. No proliferation of HLA-mismatched CD4+ T cells was observed in response to MSC or MSC-γ, whereas dendritic cells with the same HLA class II mismatch as the MSCs did stimulate proliferation of CD4 T-cells **(Figure 2A).** Next, the immunosuppressive potential of MSCs and MSC-γ was assessed in a co-culture of MSC or MSC-γ with HLA-mismatched PBLs that were activated with CD3/CD28 beads. CD3/CD28 beads induced T-cell proliferation that was inhibited by MSC-γ and to a lesser extent by non-activated MSCs **(Figure 2B)**. Non-activated MSCs significantly inhibited CD3/CD28 bead-stimulated proliferation of HLA-mismatched CD4 T-cells ($p < 0.0001$) and activation of MSCs significantly enhanced this inhibitory potential, compared to non-activated MSCs ($p = 0.008$; one-way ANOVA with Tukey's correction for multiple comparisons) (**Figure 2C**).

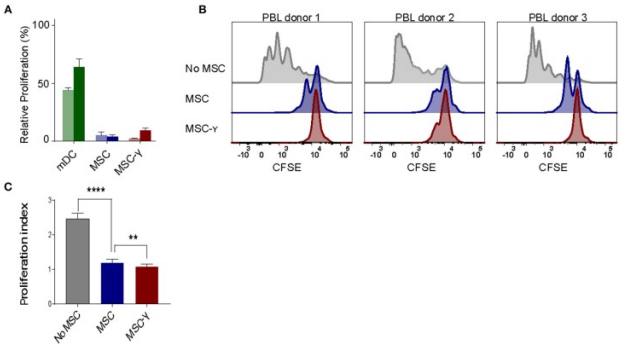

Figure 2: Alloresponse and immunosuppressive capacity of activated MSCs. (A) *A mixed lymphocyte reaction (MLR) was performed. Proliferation of CFSE-labelled PBLs from two independent donors (dark and light bar) in a co-culture with HLA-mismatched MSCs (blue bars), or MSC-γ (red bars). HLA-mismatched mDCs (green bars) were used as a positive control of proliferation of allo-reactive T-cells. Proliferation was calculated relative to CD3/CD28 bead induced proliferation of PBLs (set to 100%) (N = 2).* ***(B)*** *A suppression assay was performed with three independent PBL donors. Proliferation of CFSE-labelled PBLs was induced by CD3/CD28 beads. Histograms represent proliferation of CD4 T-cells when stimulated with CD3/CD28 beads alone (gray histograms), or in the presence of non-activated MSC (blue histograms), or MSC-γ (red histograms). The panels represent proliferation of three different allogeneic PBL donors in co-culture with one MSC donor.* ***(C)*** *This experiment was repeated three times, each time with different PBL and MSC donors. The bar graph shows the proliferation index of different allogeneic CFSE-labelled PBL donors, activated by CD3/CD28 beads, cultured with no MSC (gray), MSC (blue), and MSC-γ (red). The proliferation index is on gated CD4 T-cells. The data are presented as mean ± SD of three different MSC donors each cocultured with different allogeneic PBL donors in three independent experiments. ** p = 0.008; **** p < 0.0001, one-way ANOVA with Tukey's correction for multiple comparisons. PBL, peripheral blood lymphocyte.*

MSCs Take Up and Process Antigen, but Do Not Induce T-Cell Proliferation

We further explored whether the immunosuppressive properties of MSCs could be combined with antigen presentation. For that, besides HLA class II expression, antigen-presenting cells need to take up and process antigen (38). We tested the antigen uptake and processing capacity of MSCs by incubating with fluorescent quenched Ovalbumin protein (OVA-DQ) that only emits light once it has been taken up and proteolytically degraded in the cell. MSCs were able to take up and process OVA-DQ, as demonstrated by the detection of a fluorescent signal by both microscopy **(Figure 3A)** and flow cytometry **(Figure 3B).** Next, to test whether the uptake and processing of an antigen by MSCs could induce antigen-specific T-cell proliferation, activated MSCs expressing HLA-DR3 were pulsed with either whole protein (GAD65) or peptide ($GAD65_{339–352}$) and cocultured with HLA-DR3-restricted $GAD65_{339–352}$-specific T-cells. Neither whole protein nor peptide prepulsed HLA-matched MSC-γ induced proliferation of $GAD65_{339–352}$ specific effector T-cell clones, whereas prepulsed, HLA-matched DCs did (**Figure 3C**).

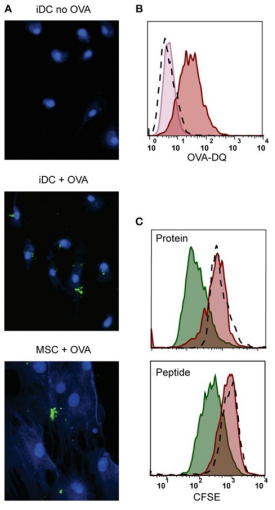

Figure 3: Antigen processing and induction of T-cell proliferation by activated MSCs. *Firstly, the antigen uptake and processing capacity of MSCs was tested. MSCs were pulsed with OVA-DQ that only fluoresces once it has been taken up and proteolytically degraded in the cell.* ***(A)*** *Fluorescence microscopy pictures of processed OVA-DQ (green) by MSCs or immature dendritic cells (iDC). Blue Mask shows nuclei and cytoplasm defining individual cells.* ***(B)*** *Histograms depict the fluorescence of processed OVA-DQ in MSCs measured by flow cytometry after 4 h incubation at 4°C (pink histogram) or 37° (red histogram) and isotype control (dashed histogram).* ***(C)*** *Consequently, the capacity of MSCs to induce proliferation of an antigen-specific T-cell was tested in a co-culture. MSCs or DCs were pulsed with GAD protein or GAD peptide and were both HLA-matched to the T-cell clone. Proliferation of T-cells was measured with CFSE dilution after 4 days of co-culture. The histograms present proliferation of CFSE-labeled GAD-specific T-cell clone upon activation with GAD-pulsed DCs (green histograms), GAD-pulsed MSC-γ (red histogram), or unpulsed MSC-γ (dashed histogram). All histograms are representative of 4 independent experiments.*

MSCs Impede Proliferation of Activated Antigen-Specific T-Cells, Imprinting the Inhibition Even After Their Removal

As activated MSCs pulsed with antigen did not induce T cell proliferation, we tested whether they modulate islet autoreactive T-cells by actively inhibiting T-cell proliferation instead. Proliferation of GAD-specific T-cells was induced by DCs expressing HLA-DR3 and GAD peptide. T-cell proliferation was assessed in the presence of DCs alone or together with activated MSCs pulsed with different concentrations of GAD peptide prior to the co-culture. The T-cell proliferation was indeed induced by DC alone and did not change in the

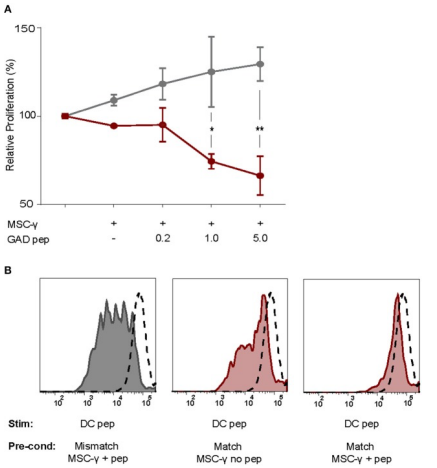

Figure 4: Antigen-specific inhibition of proliferation by activated MSCs. *The capacity of MSCs to inhibit antigen-specific proliferation of a T-cell clone was assessed.* ***(A)*** *Proliferation of a GAD-specific T-cell clone was induced by HLA-matched and peptide-pulsed DCs, which was set to 100%. HLA-mismatched MSC-γ (gray symbols) or HLA-matched MSC-γ (red symbols) that were prepulsed with increasing concentrations of GAD peptide (GAD pep) were added to the DC and T-cell co-culture. Prepulsing HLA-matched MSCs with GAD peptide significantly inhibited proliferation of a GAD-specific T-cell clone, compared to HLA-mismatched MSCs: 1 µg/mL (*p = 0.013) and 5 µg/mL (**p = 0.003). Difference was tested using a two-way ANOVA with Sidak's correction for multiple comparisons.* ***(B)*** *Conditioning experiment to test whether MSCs are needed in the co-culture to inhibit antigen-specific T-cell proliferation. GAD-specific T-cell clones were preconditioned (Pre-cond) for 24 h by HLA-mismatched (gray histogram) MSC-γ, or HLA-matched (red histogram) MSC-γ pulsed (right panel) or not pulsed (middle panel) with GAD peptide (pep). Consequently, the GAD-specific T-cell clone was harvested and stimulated (Stim) with HLA-matched and peptide-pulsed DCs. Proliferation of T-cells was measured with CFSE dilution. Proliferation of unstimulated T-cells is depicted in the dashed histogram. All histograms and graphs are representative of two independent experiments.*

presence of GAD-peptide pulsed, activated MSCs carrying HLA-DR13 that is irrelevant for these T-cells. If anything, the proliferation of T cells in the presence of pulsed MSCs with mismatched HLA tended to increase although not significantly. The stimulation of GAD-specific T cells in the presence of HLA-DR3 MSCs was reduced in a peptide-dose dependent manner, compared to HLA-DR13 MSCs (GAD peptide 1 µg/mL $p = 0.013$; 5 µg/mL $p = 0.003$; **Figure 4A**), underscoring the need for HLA-matching to induce antigen-specific T-cell inhibition by activated antigen-pulsed MSCs.

To test whether the presence of MSCs in the co-culture is necessary to inhibit the DC-induced proliferation of auto-reactive T-cells, GAD-specific T-cells were preconditioned

for 24h with MSCs that were either HLA-matched (DR3) or mismatched (DR13) with or without peptide. Next, the non-adherent T-cells were harvested carefully from adherent MSCs, washed and transferred to new co-cultures with peptide-pulsed HLA-matched (DR3) DCs. T-cell proliferation was measured by CFSE dilution after 4 days of co-culture with DCs (**Figure 4B**). When preconditioning was performed with activated and HLA-matched MSCs pulsed with peptide, proliferation of T-cells was totally abolished in the subsequent DC co-culture. In contrast, T cells proliferated when pre-conditioned with activated and peptide-pulsed DR13 MSC. Also, T cell proliferation was only marginally inhibited if the preconditioning of matched and activated MSC occurred in the absence of antigen.

In summary, only conditioning of antigen-specific T-cells by activated and HLA-matched MSCs pulsed with antigen inhibited subsequent DC-stimulated T-cell proliferation, even upon removal of the MSCs.

Discussion

MSCs have shown great promise as an immune-modulating therapy in the clinic for several diseases, but thus far they have been solely explored as an antigen-non-specific therapy (3). Combining the immunosuppressive properties of MSCs with antigen-presenting qualities would create an attractive cellular product for immune modulation. In this study, we provide *in vitro* evidence that activated MSCs can take up and process antigens and upregulate HLA class II expression, collectively granting MSCs the conditions necessary to transform into unconventional antigen-presenting cells.

We confirmed that MSC activation does not alter their hypoimmunogenic profile. Although HLA-DR expression was increased after activation, this was not accompanied by an increase in activating co-stimulatory molecules CD86 and CD80. Activation of MSCs did also not change expression of chemokine receptors CCR7 and CXCR3, implicated in migration to lymph nodes and inflamed tissues, respectively (36). Instead, activation increased the expression of immunosuppressive checkpoints such as PD-L1 and IDO, both known to endorse MSCs with immuno-modulatory capacities (39, 40). While high concentrations of IFN-γ alone activated MSCs, we now show that the cytokines secreted by antigen-stimulated T-cells could activate MSCs in a similar fashion, suggesting that inflammation *in vivo* may reinforce immunosuppressive capacity of MSCs without increasing their immunogenicity. We propose that this extended Th1 cytokine profile is more representative of an actual T-cell response to antigen than the rather excessive and selective cytokine(s) usually tested to mimic inflammation. This increase in inhibitory markers matches our findings that activation of MSCs actually reinforces their immunosuppressive capacity in a mixed lymphocyte reaction.

Non-activated MSCs are highly glycolytic (41), which has been linked to their immunosuppressive capacity (42), while mitochondrial respiration proved less important to the suppressive functionality of MSCs (41). Our findings point toward a stable metabolic phenotype in terms of mitochondrial respiration and glycolysis after activation of MSCs. Yet, non-mitochondrial respiration was increased upon activation, which could signify a more extensive usage of desaturases and detoxification enzymes (43).

Taking up and presenting antigens on HLA class II molecules is a *sine qua non* of antigen-presenting cells (38). Activated MSCs in our study were able to take up and process antigen in line with previous findings (10–12). While activation and peptide-pulsing of MSCs did not induce proliferation of peptide-specific T-cells, they were able to inhibit the proliferation of autoreactive T-cells in an antigen-specific manner. Peptide-pulsing alone did not transform MSCs into suppressive cells, as activated and peptide-pulsed but HLA-mismatched MSCs did not inhibit T-cell proliferation. We show that MSCs interfere in T cell activation induced by professional APC (i.e., DCs), as well as endorse an inhibitory effect in T cells lasting beyond their presence. Indeed, it is intriguing that a 24-h preconditioning of T-cells with MSC loaded with their antigen was sufficient to change the course of events of those T-cells in the subsequent 4 days after removal of MSCs. It should be noted that the T-cells were washed after the MSC preconditioning so the effect on T-cell inhibition cannot be accounted for by soluble factors or microvesicles of MSCs.

Our finding that HLA class II matching with the recipient is required in order to deliver adaptive immune alterations implies that the suppressive licensing by MSCs is a direct consequence of peptide presentation on the appropriate HLA restriction elements to the T-cell. This is the case for both intervening in an antigen-specific response as for preventing the induction of a response. In fact, proliferation of GAD-specific T-cells was slightly increased in case the MSC had a different HLA than the T-cell. We propose that this is due to increased presentation by dendritic cells of peptides that had leaked from peptide-pulsed MSCs during co-culture, as increasing concentrations of peptide used to pulse MSCs resulted in a slight increase in T-cell proliferation. Similar leak or “delivery” of antigen by MSCs to DCs has been reported (12). Collectively, antigen-specific immune modulation by activated MSCs was dependent upon the presence of the relevant islet peptide epitope, the appropriate HLA-DR3 restriction element for presentation of the islet epitope and showed an epitope dose-dependent increase in inhibition of T-cell proliferation. Nevertheless, matching MSCs for one HLA-haplotype with the T-cell donor was sufficient to inhibit antigen-specifically. This increases the number of potential MSC recipients in an off-the-shelf therapy, while limiting the risk of alloreactivity (13). No allo-response was provoked by MSCs *in vitro* in our studies, even when these were induced to express completely mismatched HLA class II.

In terms of underlying mechanism, our data suggests that the antigen-specific inhibition of T-cell proliferation by MSCs results from antigen presentation in HLA class II in the absence of co-stimulatory activation, similar to tolerogenic dendritic cells (34), as activated MSCs lack CD80 and CD86. Cytokine mediated modulation seems unlikely, since activation of MSCs did not affect their cytokine secretion profile. We favor the possibility of a role for the inhibitory molecules PD-L1 and IDO, which are both increased on activated MSCs and could lead to an inhibitory rather than stimulatory signal to T-cells. In concert, this may result in suppression of an adaptive (auto)immune response.

Inconsistencies have been reported with regard to adaptive features of MSCs upon activation between mice and men, and between human studies. One discrepancy was noted between mice and men that relates to MSC density, which inversely affected their antigen processing and MHC class II upregulation (44). We also found that activation and peptide-pulsing of human MSCs resulted in inhibition of T-cells, whereas Ag-pulsed and activated mouse MSCs activated T-cells (11). Their T-cell activation was CD80 dependent, whereas human MSCs do not express CD80 upon activation, which we confirm (45). In terms of inconsistencies between human studies, one report claimed that antigen presenting and stimulating qualities of MSCs were uniquely induced by low levels of IFN-γ, whereas HLA class II was decreased at higher IFN-γ levels, while we show that MSCs also express HLA class II at high IFN-γ exposure (10). Yet, our data are consistent with their observation that immune suppression would be more pronounced during severe inflammation. Furthermore, these MSCs were shown to induce antigen-specific T-cell proliferation in a short co-culture (10), while we found immune suppression. This discrepancy might be explained by duration of co-culture (18). Indeed, human MSCs were reported to inhibit T-cell activation in long-term cultures, which we confirm, whereas shorter cultures activated T-cells. To conclude, our *in vitro* data are consistent with other human studies with similar conditions and point to an antigen-specific suppressive role for MSCs, which further supports the hypoimmunogenic profile of MSCs (1, 46).

We here provide proof-of-concept that activated MSCs could take up and process antigen and inhibit proliferation of activated effector T-cells in an antigen-specific manner, without overtly increasing the immunogenicity of allogeneic MSCs. These features provide encouraging first steps in the clinical translation of the use of pre-activated MSCs as a cellular immune intervention therapy. This could pave the way to use activated HLA-haplotype matched allogeneic MSCs as immunomodulatory therapeutic cell products for intervention in adaptive immunity in autoimmune disease.

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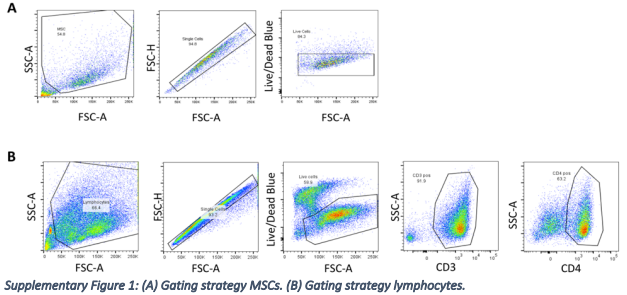
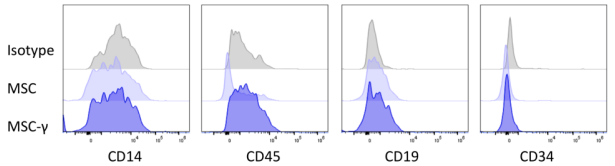
Supplementary Material

Target	Clone	Fluorochrome	Company
CD73	AD2	PE-Cy7	Biolegend
CD90	Thy-1A1	APC	R&D systems
CD105	43A3	PE	Biolegend
HLA DR	G46-6	BV605	BD Biosciences
CD80	BB1	FITC	BD Pharmigen
CD86	L307.4	PE	BD Pharmigen
CXCR3	1C6	PE	BD Pharmigen
CCR7	3D12	FITC	eBiosciences
FAS	DX2	FITC	Biolegend
ILT3	ZM3.8	PE-Cy7	Beckman Coulter
PDL1	MIH1	PDL-1	eBiosciences
IDO	Eyedio	PE	eBiosciences
CD3	UCHT1	AF700	BD Biosciences
CD4	RPA-T4	APC-Cy7	BD Biosciences
CD14	61D3	FITC	Thermo Fisher
CD19	HIB19	PerCP-Cy5.5	Biolegend
CD34	561	AF700	Biolegend
CD45	H130	BV421	BD Biosciences

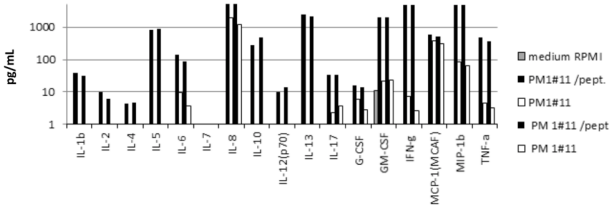
Supplementary Table 1: List of anti-human monoclonal antibodies used for MSC phenotyping



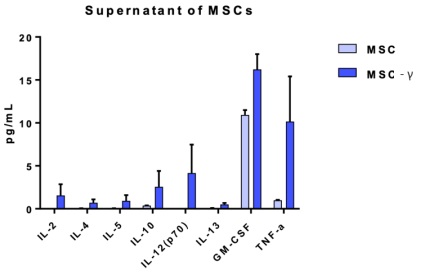
Supplementary Figure 1: (A) Gating strategy MSCs. (B) Gating strategy lymphocytes.



Supplementary Figure 2: Negative markers of MSCs. *Representative histograms of negative markers for non-activated (MSC) and activated MSCs (MSC-γ), compared to isotype controls. N= 5.*



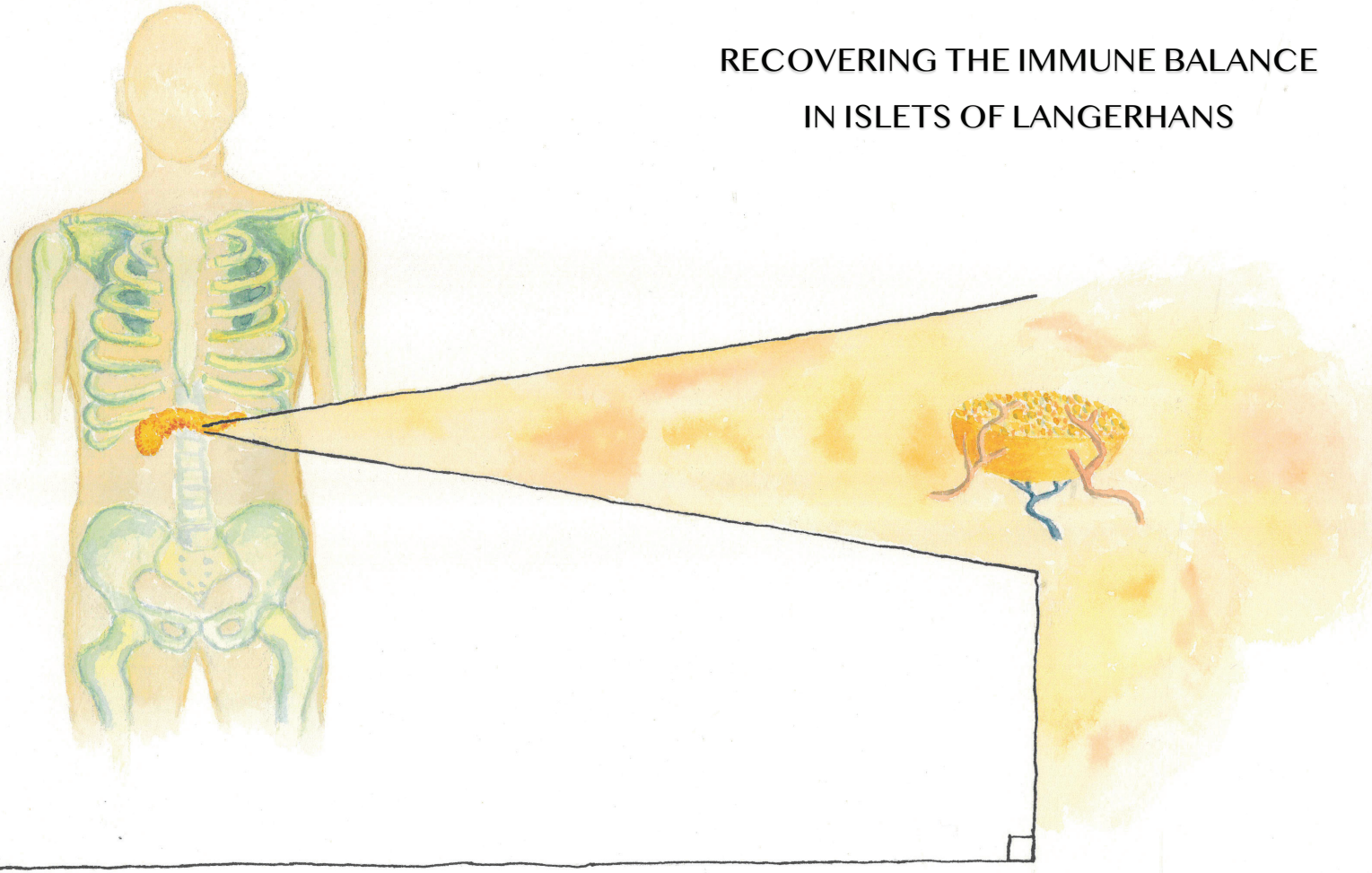
Supplementary Figure 3: Cytokine production of activated and non-activated GAD-specific T-cell clone. *Cytokine profile of GAD65 T-cell clone upon GAD65 peptide stimulation. Results are shown as result of two independent experiments. Grey bars show the cytokine concentration in media (RPMI), white bars the cytokine profile of resting GAD65 T-cells, and the black bars indicate the peptide-specific cytokines release.*



Supplementary Figure 4: Cytokine production of activated and non-activated MSCs. *Luminex assay of the supernatant of non-activated (MSC) and activated MSCs (MSC-γ). The data are presented as mean ± SD of three different MSC donors.*

PART III

RECOVERING THE IMMUNE BALANCE IN ISLETS OF LANGERHANS



Chapter 6

Intra-pancreatic tissue-derived mesenchymal stromal cells: a promising therapeutic potential with anti-inflammatory and pro-angiogenic profiles

Khiatah B, Qi M, Du W, T-Chen K, van Megen KM, Perez RG, Isenberg JS, Kandeel F, Roep BO, Ku HT, Al-Abdullah IH

Stem Cell Research & Therapy. 2019; 10:322

Abstract

Background

Human pancreata contain many types of cells, such as endocrine islets, acinar, ductal, fat, and mesenchymal stromal cells (MSCs). MSCs are important and shown to have a promising therapeutic potential to treat various disease conditions.

Methods

We investigated intra-pancreatic tissue-derived (IPTD) MSCs isolated from tissue fractions that are routinely discarded during pancreatic islet isolation of human cadaveric donors. Furthermore, whether proangiogenic and anti-inflammatory properties of these cells could be enhanced was investigated.

Results

IPTD-MSCs were expanded in GMP-compatible CMRL-1066 medium supplemented with 5% human platelet lysate (hPL). IPTD-MSCs were found to be highly pure, with > 95% positive for CD90, CD105, and CD73, and negative for CD45, CD34, CD14, and HLA-DR. Immunofluorescence staining of pancreas tissue demonstrated the presence of $CD105^+$ cells in the vicinity of islets. IPTD-MSCs were capable of differentiation into adipocytes, chondrocytes, and osteoblasts in vitro, underscoring their multipotent features. When these cells were cultured in the presence of a low dose of TNF-α, gene expression of tumor necrosis factor alpha-stimulated gene-6 (TSG-6) was significantly increased, compared to control. In contrast, treating cells with dimethyloxallyl glycine (DMOG) (a prolyl 4-hydroxylase inhibitor) enhanced mRNA levels of nuclear factor erythroid 2-related factor 2 (NRF2) and vascular endothelial growth factor (VEGF). Interestingly, a combination of TNF-α and DMOG stimulated the optimal expression of all three genes in IPTD-MSCs. Conditioned medium of IPTD-MSCs treated with a combination of DMOG and TNF-α contained higher levels of pro-angiogenic (VEGF, IL-6, and IL-8) compared to controls, promoting angiogenesis of human endothelial cells in vitro. In contrast, levels of MCP-1, a pro-inflammatory cytokine, were reduced in the conditioned medium of IPTD-MSCs treated with a combination of DMOG and TNF-α.

Conclusions

The results demonstrate that IPTD-MSCs reside within the pancreas and can be separated as part of a standard islet-isolation protocol. These IPTD-MSCs can be expanded and potentiated ex vivo to enhance their anti-inflammatory and pro-angiogenic profiles. The fact that IPTD-MSCs are generated in a GMP-compatible procedure implicates a direct clinical application.

Background

Mesenchymal stromal cells (MSCs) have the potential for treating various diseases (1). Currently, over 800 clinical trials involving MSCs have been registered (clinical-trials.gov), the majority of which are focusing on the application of MSCs to diseases of the musculoskeletal and cardiovascular systems as well as autoimmune type 1 diabetes (T1D) (2, 3). With respect to the treatment of diabetes with MSCs, some encouraging progress has been made. For example, intravenous injection of umbilical blood-derived allogeneic MSCs improved the function of pancreatic β-cells, reduced the incidence of diabetic complications, and led to insulin independence in some type 2 diabetic patients (4, 5). Autologous MSCs were used to treat individuals with T1D and lead to the preservation of C-peptide (6). For this, bone marrow-derived MSCs were aspirated from iliac crest, a procedure with substantial discomfort (6). Moreover, the administration of bone marrow-derived allogeneic MSCs together with pancreatic islets enhanced islet survival in diabetic non-human primates (7). These studies employed fetal bovine serum in the MSC culture media, which is less desirable than media that lack animal proteins, pointing to a need for alternative culture and expansion strategies.

The mechanism by which MSCs protect human islets includes the expression of anti-inflammatory and pro-angiogenic genes (8, 9). Tumor necrosis factor alpha-stimulated gene-6 (*TSG-6*) induced by TNF-α has anti-inflammatory properties (10–12). Nuclear factor erythroid 2-related factor 2 (*NRF2*) is important in enhancing islet graft survival and function (13, 14). Additionally, dimethyloxallyl glycine (DMOG), which targets prolyl-4-hydroxylase to prevent the degradation of hypoxia-inducible factor-1α (15) and upregulate vascular endothelial growth factor (VEGF) (16), could be a possible conditioning factor for improving MSC function.

MSCs have been isolated from various sites including subcutaneous adipose tissue (17, 18), bone marrow (19, 20), skeletal muscle (21), umbilical cord blood (22), ocular limbus (23), and amniotic fluid (24). Blood- and adipose-derived MSCs are widely investigated due to their accessibility, expandability, differentiability, and clinical applicability (25, 26). During the enzymatic digestion of the cadaveric pancreas, cells are liberated, together with islets, which can then be separated and characterized. In this study, we isolated MSCs from the otherwise discarded fractions of pancreatic tissue. These cells, designated as intra-pancreatic tissue-derived (IPTD) MSCs, were cultured in a GMP-grade and xenoprotein-free culture medium containing human platelet lysate and conditioned in vitro with TNF-α (27) and DMOG. Changes in gene expression, growth factor, and cytokine levels and angiogenic capacity after conditioning were determined. This study identifies a previously unappreciated fraction of the pancreatic digest as a useful source of anti-inflammatory and pro-angiogenic MSCs with possible clinical applications.

Methods

Digestion of human pancreata from cadaveric donors

Human cadaveric donor pancreata (*n* = 9) were obtained from an organ procurement organization. Cadaveric donors from which IPTD-MSCs were obtained averaged 33.8 ± 3.1 years of age, 29.8 ± 1.8 body mass index, and 5.1 ± 0.1% hemoglobin A1c (**Table 1**). Pancreata from individuals with the criteria of Donation after Cardiac Death and HbA1c > 6.5% were excluded from this study. Islet isolation was carried out in a cGMP facility at City of Hope as previously described (28, 29). Briefly, the pancreas was digested using collagenase supplemented with either thermolysin or neutral protease (28). The digested pancreatic tissues were collected in 18 250-mL conical tubes and centrifuged at 182×*g*/8°C for 3 min. Pancreatic tissue was collected, washed, and purified in a cold COBE 2991 cell processor (COBE Laboratories Inc., Lakewood, CA, USA) (30). Fractions of purified islets were collected and the IPTD-MSCs were cultured as described below.

Intra-pancreatic tissue-derived cell harvesting and culture

Enzymatic digestion of the whole pancreata released intra-pancreatic tissue and stromal cells. These cells were found to be less dense than the islets and acinar clusters. Under the standard centrifugation condition (182×*g* for 3min), which was prioritized for islets and acinar clusters, the stromal cells were located at the top layer of the conical tubes (**Figure 1**). Until now, this top layer of tissue and cells has been routinely discarded.

To test our hypothesis that IPTD-MSCs can be separated from fractions of the pancreatic tissue, we modified our standard protocol, collected and pooled the upper layer found post-centrifugation, and passed the resultant through double layers of mesh filters (500 and 300 μm) to eliminate non-cellular components (**Figure 1**). The filtered cells were then washed with CMRL-1066 culture medium and centrifuged at 727×*g*/8 °C for 3 min. The supernatant was aspirated, and the pellet was suspended in CMRL-1066 culture medium supplemented with 5% Human Platelet Lysate (hPL, Compass Biomed, MA) followed by transferring to a 50-mL conical tube. The suspended cells were centrifuged at 727×*g*/8°C for 3min. The supernatant was aspirated, and the pellet was suspended in 40 mL of CMRL-1066 medium containing 5% hPL followed by culturing in T-175 adherent flasks (ThermoFisher Scientific, Waltham, MA) for 24 h at 37°C in 5% CO_2 (**Figure 1**). Twenty-four hours later, the medium was replaced with fresh CMRL-1066 medium containing 5% hPL. Additional media changes were performed every 48 h until cells reached ~80-90% confluence.

Isolation number	Age (years)	Race	Sex	HbA1c (%)	BMI	Cause of death
Donor #1	47	C	M	4.7	31.7	CVA
Donor #2	27	H	M	5.2	30.7	T
Donor #3	39	C	M	5	30	ICB
Donor #4	17	H	M	5.2	39.4	HT
Donor #5	38	C	M	5.2	26.3	HT
Donor #6	34	C	M	4.8	33.1	HT
Donor #7	27	H	M	5	24.7	CVA
Donor #8	31	H	M	5.3	31.8	HT
Donor #9	44	AA	M	5.2	20.3	HT
Mean±SEM	33.8±3.1	NA	NA	5.1±0.1	29.8±1.8	NA

Table 1: Characteristics of donors of pancreata used for islet and IPTD-MSC cell isolation. *BMI - body mass index; HbA1c - hemoglobin A1c; C – Caucasian; H – Hispanic; CVA - cerebrovascular accident; HT - head trauma; AA - African-American; NA - not applicable.*

Bone marrow-derived MSCs

Bone marrow-derived human MSCs were obtained from healthy individuals as described (31, 32). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Board of Leiden University Medical Center (LUMC).

Characterization of IPTD-MSCs

Cell morphology

To record the growth and morphology of cultured cells, multiple pictures at different magnifications and time points were obtained using a ckx31 Olympus microscope.

Flow cytometry

After reaching 80–90% confluence, cells were dissociated with TrypLE (ThermoFisher, San Diego), washed with DPBS (Corning, Tewksbury, MA) twice, and incubated with antibodies specific for cell-surface molecules, including CD90, CD105, CD73, CD9, CD45, CD34, CD14, and HLA-DR (BioLegends, San Diego, CA), for 20 min at room temperature. In parallel, aliquots of cells were incubated with matched isotype control antibodies from the same supplier. After antibody incubation, cells were washed twice with DPBS and suspended in DPBS for flow cytometry analysis using a Sony SA3800 Spectral Analyzer (Sony Biotechnology, San Jose, CA). Data analysis was performed using Flowjo software (Tree Star, Ashland, OR). To verify the results, human bone marrow-derived MSCs were cultured

in the same medium used for IPTD-MSCs, passaged, and expanded in the same procedures for subsequent analysis.

Immunofluorescent staining for IPTD-MSCs and pancreatic tissue

Cells were cultured to 70–80% confluence and dissociated into a single-cell suspension using TrypLE as described (33). Cells and pancreatic tissue were then fixed in 10% cold formalin, prepared in a paraffin block, and sectioned. Antigen retrieval was performed using a citric acid-based antigen unmasking solution (Vector, pH 6.0). Sections were treated with protein block (Biogenex, Fremont, CA) to reduce background signal, followed by incubation with mouse anti-CD105 antibody (ready to use; Biogenex) and ALEXA 488-conjugated goat anti-mouse IgG antibody (1:200 dilution; ThermoFisher). Guinea pig anti-insulin (ThermoFisher) and ALEXA 647-conjugated goat anti-Guinea pig IgG antibodies (1: 200 dilution; ThermoFisher) were used for pancreatic tissue staining only. Fluoroshield^{TM} containing DAPI (Sigma Aldrich St. Louis, MO) was used to stain nuclei. Image acquisition was done using an Observer Z1 microscope (Carl Zeiss), with the objective lens set at 20×. Image processing was done using the Zen 2.0 software.

Multilineage differentiation of IPTD-MSCs

IPTD-MSCs at the second passage were cultured in T-75 tissue culture flasks until ~ 85% confluence. For adipogenic differentiation, IPTD-MSCs were seeded into 6-well plates and cultured in MesenCult^{TM} Adipogenic Differentiation Kit (STEMCELL Technologies, Vancouver, Canada; Cat# 05412) for 21days with media changed every 3 days. The presence of lipid droplets in cells was determined by staining with Oil Red O (Sigma, cat# O0625) 21 days after culture. For chondrogenic differentiation, IPTD-MSCs were cultured in two 15-mL conical tubes in MesenCult^{TM}-ACF Chondrogenic Differentiation medium (STEMCELL Technologies, Cat# 05455) for 24 days with media changed every 3 days. After culture, Alcian Blue (Sigma, cat#66011) was used to stain for both fresh cells and the cells fixed in paraffin sections. For osteogenic differentiation, cells were cultured in a T-75 tissue culture flask for 22days with media changed every 3 days. The osteogenic differentiation medium consisted of CMRL-1066 containing 10 mM β-glycerophosphate (Sigma, Cat# G-6251), 50 µg/ mL L-ascorbate acid 2-phosphate (Cayman, Item # 16457), 1µM of dexamethasone (Fresenius Kabi, Cat# 401780G), and 3% hPL. Differentiated cells were fixed in paraffin section and stained with von Kossa for calcium deposition. Undifferentiated IPTD-MSCs were cultured in standard culture medium lacking differentiation factors and stained with Oil Red O, Alcian Blue, or von Kossa.

In vitro expansion of IPTD-MSCs

T-175 flasks of ~80% confluent passage-3 cells were washed twice with DPBS, and 5 ml of TrypLE enzyme was added to each flask. The cells were incubated at 37 °C for 5-10 min to

dissociate adherent cells, and 10 ml of CMRL-1066 medium was added to terminate enzyme digestion. Cells were collected in 15-ml tubes for centrifugation at 528×*g* for 3min. The cell pellet was suspended in 5ml CMRL-1066 with 5% hPL and vortexed. A sample of cells was mixed in a 1:1 ratio with 0.4% trypan blue (ThermoFisher), from which 20 µL was placed on a counting slide (Cellometer SD100, Nexcelom Bioscience, San Diego, CA) and counted using a Cellometer Auto T4 (Nexcelom Bioscience, San Diego, CA). To further characterize the growth capabilities of these cells, we performed subcultures by placing 5×10^4 cells in T-25 flasks for 72 h at 37°C and 5% CO_2. Some cells were grown in CMRL-1066 culture medium alone and others in CMRL-1066 culture medium supplemented with 5% hPL. Culture medium was replaced once during this period. At the end of the culture, cells were dissociated and counted as above. This process was then repeated. After each passage, the cell count was multiplied by the dilution factor to calculate the total number of cells per passage.

Cryopreservation of IPTD-MSCs

Isolated IPTD-MSCs (at passage 3) were cultured to ~80% confluence, dissociated into single cells with Try-pLE, collected, and counted. Aliquots of 1×10^6 cells were divided into cryopreservation tubes, suspended in 10% DMSO in CMRL-1066 medium, and stored at −80°C in a Mr. Frosty Freezing apparatus containing 100% isopropyl alcohol (ThermoFisher). Using this method, IPTD-MSCs were stored for 9 months. The cells were then thawed rapidly in a 37°C water bath, washed with DPBS, and cultured in T-75 tissue culture flasks using CMRL media with 5% hPL. After 48 h, the cells were noted to be ~80% confluent and were subjected to subsequent analyses. Viability was assessed with trypan blue.

In vitro treatment of IPTD-MSCs with TNF-α and DMOG

Recombinant human TNF-α protein (R&D Systems, Minneapolis, MN) was reconstituted in research-grade water (Hospira, Lake Forest, IL) to a concentration of 100 ng/mL, aliquoted, and stored at −20 °C. Dimethyloxallyl glycine (DMOG; Cayman Chemicals, Ann Arbor, MI) was dissolved in water to yield a stock solution of 57.1 mM, aliquoted (100 µL), and stored at −80 °C. IPTD-MSCs were incubated in T-25 flasks in 5ml of CMRL-1066 medium supplemented with 5% hPL until ~50% confluent. Cells were cultured for 24 h in CMRL 1066 medium, or medium containing 10 ng/ml TNF-α, 1 mM DMOG or 1 mM DMOG and 10 ng/ml TNF-α. Following treatment, cells were collected in 1.7-ml Eppendorf tubes and stored in RLT buffer (Qiagen, Germantown, MD) at −80 °C for future preparation of cDNA.

Quantitative real-time PCR

The TaqMan Gene Expression Assay system (Thermo-Fisher Scientific) was used to quantify *ß-ACTIN*, *TSG-6*, *NRF2*, and *VEGF* mRNA levels. Total RNA was extracted using a Qiagen Mini Kit (Cat. No. 51306) and converted into cDNA. Real-time quantitative PCR was run in duplicate on a ViiA™ 7 Real-Time PCR System with a 384-well block (ThermoFisher Scientific). Thermal cycles were programmed for 20 s at 95°C for the initial denaturation, followed by 45 cycles of 120 s at 95°C for denaturation, 30 s at 60°C for annealing, 60 s at 72°C for extension, and a final extension at 72°C for 10min. All PCR runs were performed with negative (water) and positive controls. *ß-ACTIN* was used as an internal control to quantify relative gene expression.

Cytokine assay

Supernatants from cells cultured for 24 h under various conditions (medium alone, or medium plus DMOG, TNF-α, or DMOG + TNF-α) were collected, and cytokine analysis performed using a Luminex assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The following growth factors/cytokines were measured: VEGF, IL-6, IL-8, MMP-9, MCP-1, MMP-2, IL-4, IL-10, and IL-1β. Samples were measured in duplicate.

In vitro angiogenesis assay

Angiogenic capacity was assessed by quantifying endothelial tube formation (34). Human umbilical vein endothelial cells (HUVECs) (Cell Applications Inc., San Diego, CA; Cat# 200p-05n) between passages 2 and 6 were cultured in a standard medium. Cells (1×10^5 cells/well) were plated in 24-well plates (Fisher, Cat # 930186) coated with Matrigel (Corning, Cat# 356234), and incubated for 30 min to allow cell attachment. Supernatants (150 µL/well) from control and stimulated IPTD-MSCs (DMOG, TNF-α, or DMOG + TNF-α) were added to obtain a total volume of 300µL per well. Plates were then incubated at 37°C, 5% CO_2 for 24h. At 4 and 24h, the wells were visualized using a Leica microscope and representative photographic images were obtained. Total endothelial tube number and tube length were determined using ImageJ software (NIH, Bethesda, MD).

Statistical analysis

Data was analyzed with GraphPad Prism software (GraphPad Software 8.0, La Jolla, CA). ANOVA one-way analysis of variance was used to compare multiple experimental groups followed by the Tukey multiple comparisons test to compare the mean values between any two groups. All the values were expressed as mean ± standard error of mean (SEM). For all the tests, $p < 0.05$ was considered significant.

Results

A chemically defined medium supports the growth IPTD-MSCs

To develop a GMP-compatible culture medium, we tested whether hPL could support the growth of IPTD-MSCs in the absence of fetal bovine serum. Under phase-contrast microscopic evaluation, IPTD-MSCs displayed elongated and spindle shapes (**Figure 2A**), a morphology consistent with the classic MSCs derived from other tissues (35). Next, we dissociated and replated IPTD-MSCs multiple times and found that the spindle shape morphology was preserved throughout numerous passages (**Figure 2B**). IPTD-MSCs were cryopreserved for 9 months, thawed, and cultured, and again the cell morphology remained stable (**Figure 2C**).

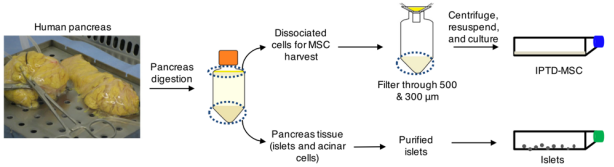


Figure 1: A schematic diagram showing the steps for isolating in a cGMP facility intra-pancreatic tissue-derived cells during human islet isolation.

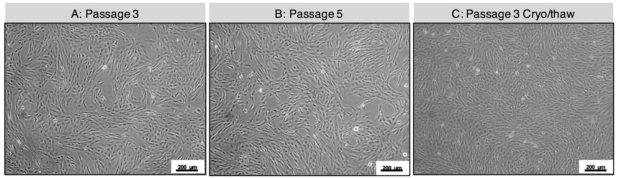


Figure 2: IPTD cells resemble MSCs in culture and can be cryopreserved. *Phase contrast microscopy of IPTD-MSCs cells cultured in CMRL-1066 medium supplemented with 5% hPL.* ***(A)*** *Passage 3 cell culture on day 3;* ***(B)*** *passage 5 cell culture on day 3;* ***(C)*** *passage 3 cells after 9 months of cryopreservation, thawing, and culture on day 3. Representative images are presented. Scale bar, 200 µm.*

hPL is required for the growth of IPTD-MSCs

We tested whether hPL is essential for the growth of IPTD-MSCs by removing it from the culture medium and passaging cells for three generations. IPTD-MSCs grown in hPL-repleted medium expanded an average of 10.8-fold, while those cultured in medium lacking hPL showed minimal to no expansion (**Figure 3**), suggesting that hPL is required for the expansion of IPTD-MSCs in vitro.

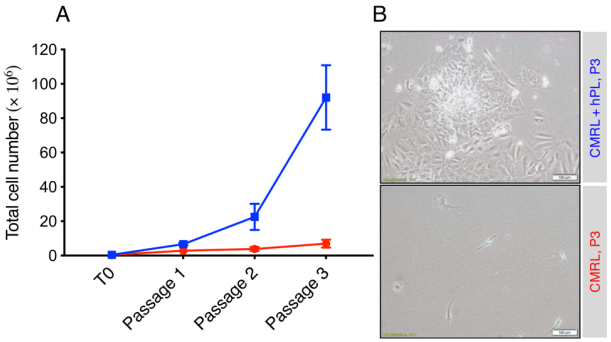


Figure 3: The growth of IPTD-MSCs is enhanced by a culture medium supplemented with hPL. ***(A)*** *Total cell numbers during expansion under the designated culture conditions. hPL was essential for the expansion of cells in vitro. The results shown were from three different tissue donors;* ***(B)*** *A representative photomicrograph of passage 3 cells cultured in CMRL-1066 culture medium with (green) and without (red) 5% hPL. Scale bar, 100 µm.*

IPTD-MSCs display classic MSC cell-surface markers

To test whether IPTD-MSCs express known markers found on MSCs isolated from bone marrow and other organs (36), we performed flow cytometry analysis. We first confirmed cell-surface marker expression using bone marrow-derived MSCs. As expected, the majority of bone marrow-derived MSCs expressed CD90, CD105, CD73, and CD9 and showed minimal to no expression of CD45 (pan-leukocytes), CD34 (hematopoietic cells), CD14 (macrophages), and HLA-DR (antigen-presenting cells) (**Figure 4A**).

IPTD-MSCs were passaged 3 times, dissociated into a single-cell suspension and stained with the above-mentioned antibodies. Compared to isotype-control staining, the vast majority of IPTD-MSCs stained positive for CD90 (99.2 ± 0.3%), CD105 (99.8 ± 0.2%), CD73 (99.6 ± 0.3%), and CD9 (86.8 ± 2.6%) (**Figure 4A**). Minimal expression of CD45 (0.3 ± 0.2%), CD34 (0.3 ± 0.0%), CD14 (1.5 ± 0.8%), and HLA-DR was found (**Figure 4A**). Expression of CD105 on the cell surface of IPTD-MSCs at passages 3 and 5 was further visualized using immuno-fluorescent staining (**Figure 4B**). Taken together, IPTD-MSCs expressed classic positive and lacked negative markers for MSCs, suggesting that they reside within the MSC family of cells.

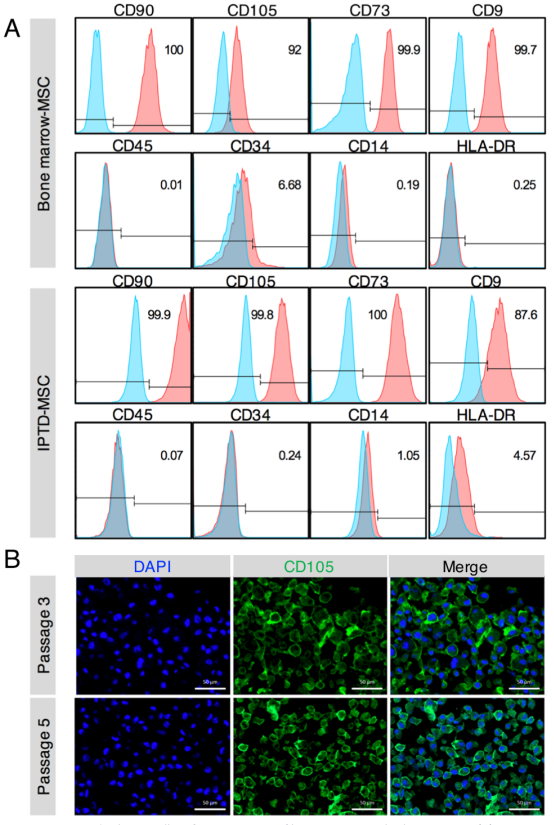


Figure 4: IPTD-MSCs display a cell-surface protein profile consistent with classic MSCs. (A) *Flow cytometry analysis of cell-surface protein expression of bone marrow-derived MSCs and IPTD-MSCs. Data are representative of four pancreas donors.* ***(B)*** *Immunofluorescent staining for CD105 protein expression (green) in paraffin sections of IPTD-MSCs (passages 3 and 5) grown in the presence of hPL. Scale bar = 50 µm. DAPI stains nuclei.*

CD105⁺ cells localize in the pancreas near insulin-expressing cells

To rule out the possibility that the ex vivo IPTD-MSC growth and expansion was due to in vitro selection or artifact, we examined whether CD105⁺ cells were present in the endogenous pancreas. Pancreatic tissue sections were co-stained with CD105 and insulin. CD105⁺ cells were detected in the pancreatic tissue and were located adjacent to the insulin-expressing islets (**Figure 5**). This result confirms the existence of CD105⁺ cells in the adult human pancreas.

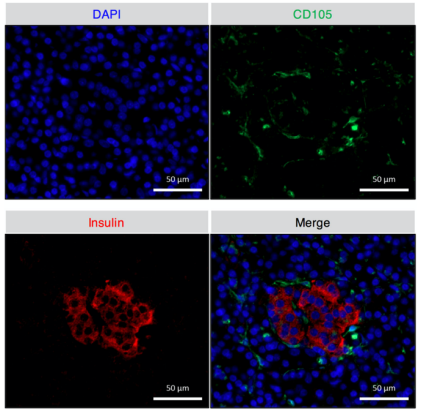


Figure 5: Cells expressing CD105 are found in non-digested pancreatic tissue. *Double immunofluorescent staining for CD105 (green) and insulin (red) revealed that CD105-positive cells are present in the pancreatic tissue and located adjacent to the insulin-expressing islets. Photomicrographs were obtained using a Z1 microscope (Carl Zeiss) at flourescence wavelengths of 488 nm (CD 105) and 647 nm (insulin). Images are representative of 3 separate experiments. Scale bars, 50 µm.*

IPTD-MSCs have potential to differentiate into multiple cell lineages in vitro

A typical feature of MSCs is the ability to assume lineage-specific cell phenotypes after exposure to certain growth factors. IPTD-MSCs were exposed to adipogenic, chondrogenic, and osteogenic growth conditions. Under these differentiation conditions, IPTD-MSCs were found to give rise to the appropriate lineage-associated phenotypes, including cells positively stained for Oil Red O (adipocytes), Alcian Blue (chondrocytes), or von Kossa (osteoblasts) (**Figure 6**). In contrast, undifferentiated IPTD-MSCs showed no lineage-specific staining.

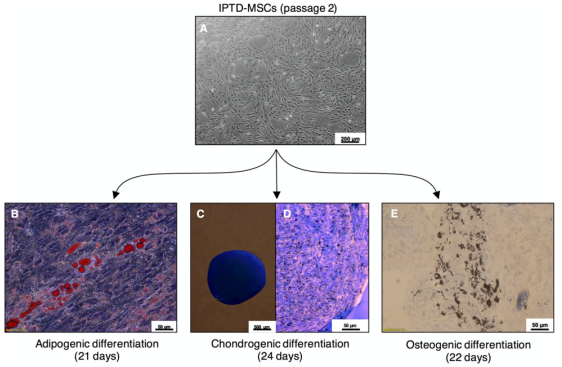


Figure 6: IPTD-MSCs can be differentiated into adipogenic, chondrogenic, and osteogenic cells. (A) *Passage 3 of IPTD-MSCs prior to differentiation.* ***(B)*** *Lipid droplets are detected after staining with Oil Red O indicating that IPTD-MSCs are undergoing adipogenic differentiation 21 days post culture.* ***(C)*** *The presence of cartilage is confirmed after staining with Alcian Blue showing in dark blue color.* ***(D)*** *Calcium deposition is detected in paraffin-fixed tissue section of the cells cultured in chondrogenic media after staining with Alcian Blue.* ***(E)*** *Calcium deposition is detected in paraffin-fixed tissue section of the cells cultured in osteogenic medium after staining with von Kossa.*

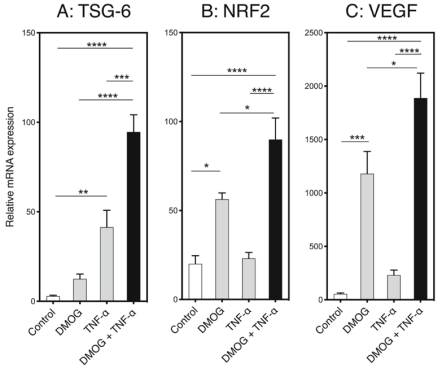


Figure 7: IPTD-MSCs treated with TNF-α and DMOG display increased mRNA levels of anti-inflammatory and pro-angiogenic genes. (A) *TSG-6,* ***(B)*** *NRF2, and* ***(C)*** *VEGF mRNA levels from IPTD-MSCs treated with TNF-α (10 ng/mL) and/or DMOG (1 mM). Three independent donors were tested. The data are expressed as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

TNF-α and DMOG upregulate immune-regulatory and angiogenic genes in IPTD-MSCs

Next, we tested whether IPTD-MSCs were amenable to in vitro conditioning. IPTD-MSCs were stimulated with TNF-α and/or DMOG, and the expression of known immune-modulating and angiogenic genes was determined. Consistent to previous findings (27), *TSG-6* gene expression levels were significantly increased in cells treated with 10 ng/ml TNF-α ($p < 0.01$) but not DMOG alone (**Figure 7A**), compared to control. Addition of DMOG to TNF-α further increased the expression of TSG-6 ($p < 0.0001$) (**Figure 7A**). *NRF2* and *VEGF* expression were significantly increased when cells were treated with DMOG ($p < 0.05$ and $p < 0.001$ respectively, Fig. 7b, c) but not TNF-α alone, compared to control. Addition of DMOG to TNF-α further enhanced the expression of *NRF2* and *VEGF* ($p < 0.0001$, **Figures 7B, C**). These results suggest that, while TNF-α and DMOG display divergent effects, the combination of the two best enhances in IPTD-MSC genes that are known to modulate immune responses and angiogenesis.

TNF-α and DMOG alter growth factors and cytokines released by IPTD-MSCs

To further characterize IPTD-MSCs, we examined proteins released from these cells. IPTD-MSCs were cultured for 24 h in the presence of exogenous TNF-α, DMOG, or both, and the resulting culture media were examined by Luminex assay. Compared to control, stimulation of IPTD-MSCs with DMOG alone enhanced the secretion of VEGF, IL-6, IL-8, and IL-4 (**Figure 8**; comparing the 1st to the 2nd bars). Stimulation of IPTD-MSCs with the combination of DMOG plus TNF-α enhanced the secretion of VEGF, IL-6, and IL-4 (**Figure 8**; comparing the 1st to the 4th bars), while the stimulation of IPTD-MSCs with TNF-α alone did not have an effect on any of the cytokines examined compared to controls. The addition of TNF-α to DMOG enhanced the secretion of IL-6 and IL-4 (**Figure 8**; comparing the 2nd to the 4th bars). Levels of MCP-1 were reduced in the conditioned media of IPTD-MSCs treated with DMOG or DMOG plus TNF-α, but not with TNF-α alone. Levels of MMP-9, MMP-2, and IL-10 were not changed in response to various conditioning while L-1β was undetectable.

Conditioned medium from IPTD-MSCs stimulated with DMOG promotes angiogenic activity of endothelial cells

Endothelial cell tube formation is an acknowledged angiogenic metric indicative of cell migration, adhesion, and re-organization. To test this, HUVECs were exposed to conditioned media from IPTD-MSCs stimulated with TNF-α, DMOG, or both. Four hours post-plating, HUVECs treated with various IPTD-MSC conditioned media displayed similar morphology without significant difference in tube number or length, regardless of the source of the media. By 24 h, endothelial tube formation was apparent (**Figure 9A**). HUVECs incubated with media from IPTD-MSCs treated with DMOG (21.0 ± 2.0, $p < 0.01$)

or DMOG + TNF-α (26.0 ± 2.0, $p < 0.01$) displayed increased numbers of cell tubes, compared to control (3.5 ± 0.5) (**Figure 9B**). Similarly, tube length was significantly higher in HUVECs exposed to IPTD-MSC conditioned media stimulated with DMOG (28.6 ± 9.9 mm, $p < 0.05$) or DMOG + TNF-α (43.6 ± 0.8 mm, $p < 0.05$), compared to control (10.3 ± 1.0 mm) (**Figure 9B**). TNF-α by itself had no effect on tube number or length, and TNF-α did not enhance the effects of DMOG (**Figure 9B**; comparing the 2nd and the 4th bars), suggesting that DMOG is the sole stimulant to enhance IPTD-MSC-mediated angiogenesis in vitro.

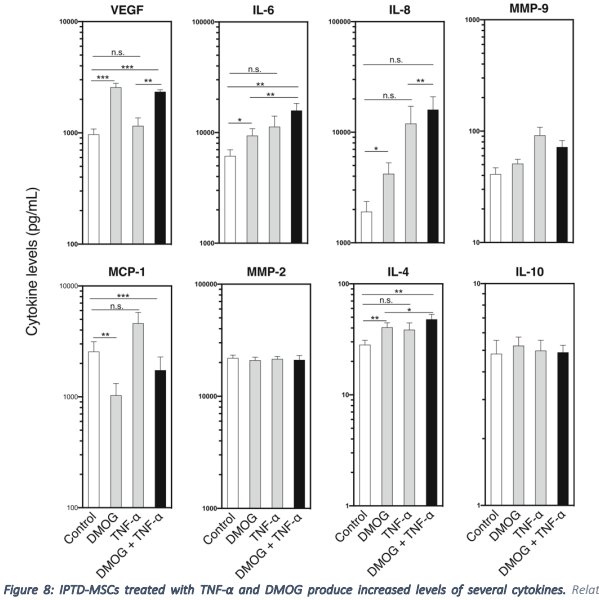


Figure 8: IPTD-MSCs treated with TNF-α and DMOG produce increased levels of several cytokines. *Relative changes of the indicated cytokines and growth factors found in medium from conditioned and control IPTD-MSCs. Y-axis is logarithmic. Four independent experiments were performed. The data are expressed as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s. not significant.*

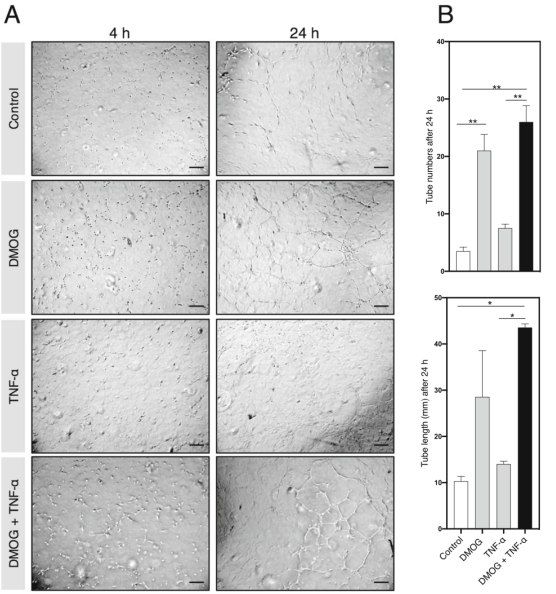


Figure 9: IPTD-MSC-derived media stimulates endothelial tube formation. (A) *Representative photographs of endothelial cells cultured and treated with medium from conditioned and control IPTD-MSCs at 4 and 24 h post-culture. Scale bar, 500 μm.* ***(B)*** *Quantification is presented as total tube number and total tube length after 24 h. Duplicate samples were performed. Representative microscopic images are presented.*

Discussion

We identified a MSC population that resides within pancreatic tissues, which can be separated during islet isolation. We named these cells intra-pancreatic tissue-derived (IPTD)-MSCs, in agreement with the recent call for nomenclature of MSCs in relation to their tissue of origin (37–39). In culture, IPTD-MSCs displayed features similar to classic bone marrow- or umbilical cord blood-derived MSCs, including adherence to culture-grade plastic surfaces, spindle-shaped morphology, expression of appropriate surface markers (positive for CD90, CD105, and CD73, and negative for CD45, CD34, CD14 and HLA-DR), and capacity for proliferation and multilineage differentiation. Furthermore, when IPTD-MSCs were treated with a combination of TNF-α and DMOG, we observed (1) increased mRNA levels of *TSG-6*, *NRF2*, and *VEGF*; (2) increased secretion from IPTD-MSCs

of VEGF, IL-6, IL-8, and IL-4; (3) decreased secretion of MCP-1; and (4) enhanced endothelial cell tube formation. Together, these results suggest IPTD-MSCs conditioned by TNF-α and DMOG have anti-inflammatory and pro-angiogenic potential.

The cell population, isolation, and culture method of IPTD-MSCs we described herein have both differences and similarities over other previously published MSCs (40). In this study, cells were isolated from intra-pancreatic tissue as a part of islet isolation procedure from a single donor. IPTD-MSCs were harvested from an otherwise discarded component after routine pancreatic digestion and islet isolation. The GMP-compatible protocol used for culturing these cells led to the production of large numbers of highly purified MSCs. We deliberately selected CMRL-1066 as the base medium to propagate IPTD-MSCs because CMRL-1066 is routinely used to culture islets for transplantation, thus reducing the burden for future clinical translation. Additionally, we eliminated animal products in culture media by using hPL, which will lower the risks of infection, allergic reactions, and product variability. Similar to MSCs derived from other tissue sources, IPTD-MSCs are capable of differentiation into adipocyte, chondrocyte, and osteo-blast lineages, demonstrating the multi-lineage potential of IPTD-MSCs.

This study demonstrates an approach that allows for harvesting islets and IPTD-MSCs simultaneously from a single donor under GMP conditions, facilitating direct clinical application. Harvesting IPTD-MSCs during human islet isolation makes the quality evaluation of isolated cells rapid and reliable and suggests opportunities for immediate clinical applications. Previously, autologous bone marrow-derived MSCs have been used simultaneously in living-related kidney transplant recipients (41). Moreover, we expanded bone marrow-derived MSCs in the same medium of CMRL-1066 supplemented with hPL and found that these MSCs were similar in phenotype and characteristics compared to IPTD-MSCs, suggesting that our medium could be used to isolate MSCs from other tissue sources. IPTD-MSC culture medium used in this study is xenoprotein-free and cGMP-compatible. The isolated IPTD-MSCs were expandable and can be produced in large scale using this culture medium. Conventionally, fetal bovine serum is supplemented in selected culture media to promote the growth of MSCs from different tissue sources (42). However, the use of non-human serum to culture cells carries the potential of transmitting infectious agents (43), immunizing effects (44), and lot-to-lot variability. In this regard, human platelet lysate has been used to replace fetal bovine serum for clinical-scale MSC expansion (45). In these studies, hPL was supplemented in minimal essential medium (MEM) to culture MSCs. In the current study, we used hPL to supplement the CMRL-1066 that has been optimized for human islets culture, and the culture system employed herein allows for optimum survival of IPTD-MSCs. This is important since a single medium system can be used for both cell sources to facilitate co-transplantation of islets and IPTD-MSCs in future studies.

This study also highlights the benefit of harvesting multiple cell types from tissue fractions of a single donor organ as part of the islet isolation procedure. It is conceivable that immunophenotypic characterization and identification of additional novel cell types residing within this tissue fraction would be valuable to study pancreatic pathophysiology arising from various diseases.

MSCs are known to reduce inflammation and enhance healing, and these functions can be further manipulated ex vivo to enhance capacities for cell therapies. Compared to control, we found that IPTD-MSCs exposed to a combination of TNF-α and DMOG, compared to single reagents, exerted a better overall outcome. Except for *TSG-6* expression, no other molecules, including the secreted factors examined in this study, were affected by TNF-α treatment alone. In contrast, DMOG alone was able to induce *NRF2*, *VEGF* expression, as well as the secretion of VEGF, IL-6, IL-8, and IL-4. These results demonstrate a dominant effect of DMOG over TNF-α. However, TNF-α was able to augment the effects brought by DMOG in increasing the expression of *TSG-6*, *NRF2*, and *VEGF* and enhancing secretion from IPTD-MSCs of IL-6, IL-8, and IL-4. Regardless, the combination of TNF-α and DMOG appeared to be optimal for the examined outcomes, including the expression of *TSG-6*, *NRF2*, and *VEGF*; secretion of VEGF, IL-6, IL-8, and IL-4; and endothelial tube formation. To the best of our knowledge, this is the first study to show a beneficial effect on MSCs by conditioning with the combination of TNF-α and DMOG.

IL-4 levels were significantly increased by the combination of DMOG and TNF-α as compared to the control or DMOG alone, whereas IL-10 production was unchanged. This is in line with previous reports demonstrating that MSCs do not secrete IL-10, but stimulate other immune cells to secrete this cytokine (46). MCP-1 (monocyte chemoattractant protein-1) is often increased upon treatment with inflammatory cytokines. We found that treatment of IPTD-MSCs with DMOG and TNF-α led to a reduction of MCP-1. Taken together, our results show the production of anti-inflammatory molecules in IPTD-MSCs. It remains to be determined if these human cells will provide protection in inflammatory settings.

VEGF, IL-6, IL-8, and MMP-9 are known pro-angiogenic factors (47–51), which may be responsible for the observed enhancement of endothelial cell tube formation. Upregulation and secretion of pro-angiogenic factors are important for several reasons: (i) MSCs from individuals with diabetes showed lowered angiogenic capacity (52) than those from individuals without diabetes, although another study reported that MSCs isolated from the bone marrow of T1D donors were phenotypically and functionally similar to those isolated from healthy individuals (53); (ii) treatment of islets with the iron chelator deferoxamine stabilized HIF-α and enhanced islet VEGF levels (54); and (iii)

treatment with exogenous VEGF improves islet engraftment (55) and β-cell mass (56), in part through increased angiogenesis. Islet survival and function post-transplantation are adversely impacted by hypoxia (57). Thus, processes that render islets hypoxia-resistant, such as increasing VEGF expression and secretion, should have beneficial effects in islet transplantation. The fact that the combination of DMOG and TNF-α also enhances *TSG-6*, *NRF2*, and *VEGF* gene expression from bone marrow-derived MSCs highlights the potential use of DMOG and TNF-α to condition MSCs other than IPTD-MSCs. Further, the upregulation of TSG-6 in both IPTD-MSCs and bone marrow-derived MSCs underscores the concept of employing TSG-6 as a marker of anti-inflammatory capacity (58).

An interesting finding was the proximity of MSCs to islets within the pancreatic tissue, implying a possible role for these MSCs in protecting islets from metabolic stress and inflammation. Besides the potential anti-inflammatory and pro-angiogenic effects of IPTD-MSCs, future studies will explore whether extracellular vesicles (EV) (59) secreted by these cells are more effective at limiting auto-immune diseases such as T1D and uveoretinitis (60).

Conclusion

In summary, the simultaneous isolation of human islets and intra-pancreatic tissue-derived MSCs was demonstrated. These IPTD-MSCs can be expanded in a clinically applicable culture system and potentiated ex vivo in their anti-inflammatory and pro-angiogenic properties. Such IPTD-MSCs, together with the islets originating from the same donor organ, may enhance islet transplantation outcome and other potential clinical applications.

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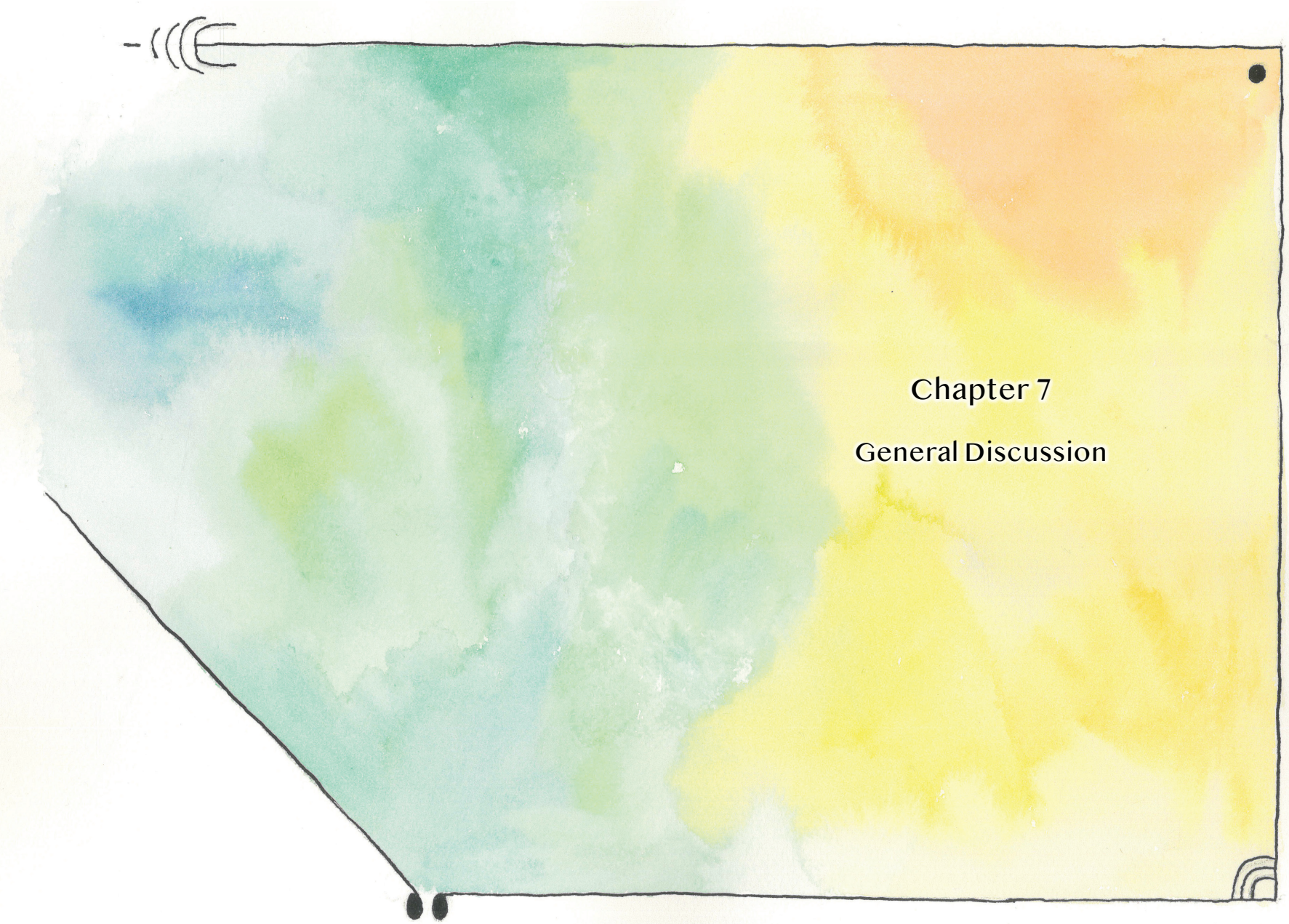
PART IV

DISCUSSION & SUMMARIES



Chapter 7

General Discussion

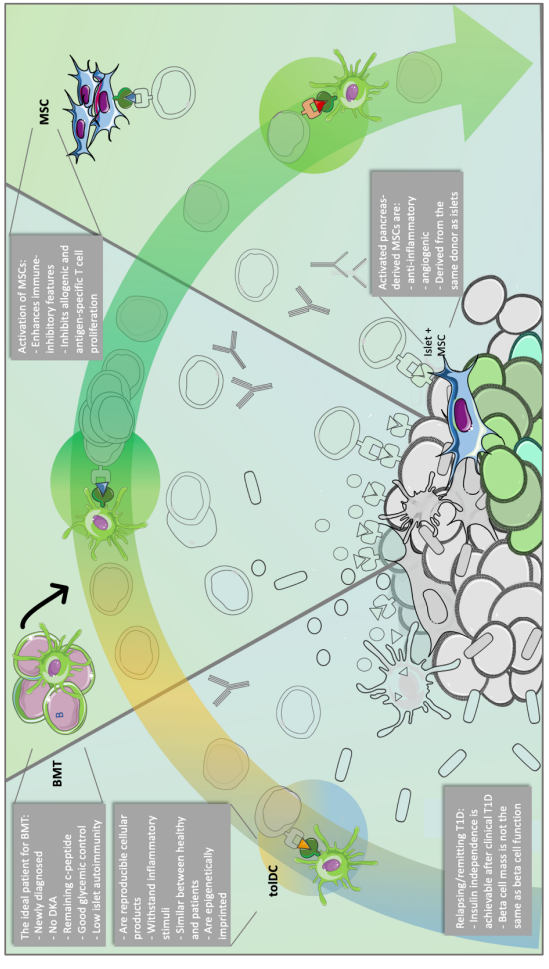
General Discussion

In this thesis, a spectrum of T1D therapies is explored: from immune suppression to immune modulation and finally therapy of the islets of Langerhans (**Figure 1**). In this discussion, five fundamental challenges regarding the development of T1D therapies will be discussed and followed by how I envision the future of these therapies.

Five challenges in discovering and implementing type 1 diabetes therapies

1. Insulin replacement is not the answer

The tragedy and blessing of T1D is that much of the general public thinks it already has a cure, namely insulin. Indeed, without insulin T1D would be a fatal disease. However, T1D Exchange data from 2014 showed that less than one in three patients over 25 years old reached their target HbA1c of 7% or less, and data from 2016 to 2018 suggest no improvements (https://t1dexchange.org/), despite the recent advances in insulin pumps and glucose sensors. Moreover, data from the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) trial demonstrated that even with intensive insulin therapy, long-term fatal complications cannot be completely prevented as small excursions out of target HbA1c range can have long term consequences, referred to as metabolic memory (1). As such, insulin does not modify the silently progressive course of T1D with its many fatal complications, such as cardiovascular disease and nephropathy. Furthermore, the longer the duration of T1D, the more prone a patient becomes to suffering from hypoglycemia unawareness with dangerous severe hypoglycemia episodes (2, 3). Therefore, when one correctly categorizes T1D as a disease with fatal risks and a loss of life-expectancy of more than ten years compared to the general population without T1D (4-6), it is hard to understand that many drugs are developed and approved for other milder auto-immune diseases, but not for T1D. The auto-immune skin disorder psoriasis, for instance, is approved for several anti-TNF-α antibodies, anti-IL-12/IL-23 and anti-IL-17A, all with various side effects including increased incidence of lymphoma (7-9). Similarly, rheumatoid arthritis (RA), an auto-immune disorder affecting the joints, is treated with TNF-α blockers, kinase inhibitors and methotrexate, of which the latter two are anti-cancer drugs (10). Although the former makes the patient more prone to infections, including tuberculosis, and in approximately 35% of patients the treatment is or becomes ineffective (11, 12), still patients and doctors are willing to take that risk. Perhaps the difference in these auto-immune disorders is that they present visible or tangible substrates of autoimmune attack (the skin for psoriasis and the inflamed joints for RA) and additionally causes pain (RA), which is an unambiguous and strong incentive for drug therapy. T1D lacks all of these



easily identifiable or measurable characteristics. Indeed, T1D lacks effective biomarkers to track disease activity, especially in terms of the amount of β cell destruction (13, 14). In a sense, after initial diagnosis, the pathogenesis and progression of T1D becomes invisible and injecting insulin becomes part of the patient's daily routine. Consequently, the urgency for the discovery of additional treatments becomes less obvious.

I propose a revision of the T1D staging system to reinstall this urgency (**Table 1**). At the moment, T1D is divided into four stages, in which stage 3 is clinical diagnosis and stage 4 is long-standing disease (15). The first three stages last up to 15 years in 80% of T1D patients diagnosed in childhood (16), though these stages could possibly last much longer in T1D patients diagnosed in adulthood (>60% of total T1D cases) (17). The last stage, however, could potentially last 50 years. In fact, there is not much information on diagnosing stage 4 T1D. Stage 4 was neither mentioned in the main paper announcing the novel staging system (15); nor in ADA's latest classification of T1D (18). Hence, this last stage begs for reconsideration. After revision, the new stage 4 could coincide with diagnosing the first microvascular complications (micro-albuminuria, non-proliferative retinopathy, peripheral neuropathy) (**Table 1**). This will include around one in three T1D patients after 10 years (19) and almost 90% of T1D patients approximately 20 years after the onset of stage 3 (20). Almost all patients still secrete c-peptide after a mixed meal tolerance test during the 20 years following clinical T1D diagnosis, indicating remaining functional β cell mass (21). Eventually this could distinguish T1D patients that could qualify for interventional immunotherapy from patients that need β cell replacement therapy. However, long-standing T1D has a much longer and divergent timeline than is appreciated in the current 4-stage system. Therefore, I propose to add two more stages.

Stage 5 would start when already-diagnosed complications progress (macro-albuminuria, proliferative retinopathy, diabetic foot disease, or angina pectoris) (**Table 1**), demarcating a transition of eligibility from immunotherapy-focused intervention trials to β cell replacement trials. Within 20 years after initiation of stage 3 almost 15% of T1D patients will be included in this stage because of progression to proliferative retinopathy and 4% because of development of macro-albuminuria. Of course, these percentages are greatly dependent on glycemic control (HbA1c). To illustrate this, 51% and 23% of T1D patients

Figure 1: Take-home Messages of Parts I, II, and III. *In this thesis, five papers describing different therapies have been discussed. Firstly, the relapsing/remitting T1D case report taught us that insulin independence can be reached even after T1D diagnosis. This suggests that beta cell mass is not equal to function and that beta cells could be recovered to secrete insulin again. Secondly, autologous bone marrow transplantation (BMT) resulted in insulin independence and patient characteristics for optimal effect were identified. Furthermore, cellular therapies that were studied were tolerogenic dendritic cells (tolDCs) and mesenchymal stromal cells (MSCs). TolDCs were stable and reproducible cellular products that were similar between healthy and T1D patients, which will expedite its use in the clinic. MSCs proved to be immune inhibitory and antigen-specific. When derived from the pancreas, MSCs could be co-transplanted with islets to improve islet transplantation. Created in Biorender.com.*

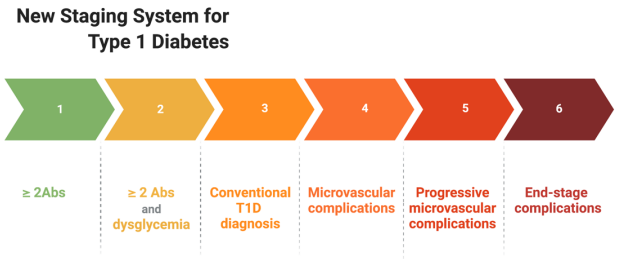


Table 1: Diagnostic Criteria for the Revised Stages of Type 1 Diabetes. *The new staging system for type 1 diabetes has 6 stages in total. The first 3 stages are identical to the staging system as brought forward by TrialNet. Stage 4 is diagnosed once patients show signs of microvascular complications, such as micro-albuminuria, non-proliferative retinopathy, and peripheral neuropathy. Stage 5 is diagnosed when these complications progress into macro-albuminuria, proliferative retinopathy, diabetic foot disease, or angina pectoris, but is not limited to these. Finally, stage 6 is diagnosed by an end-stage complication, such as end-stage renal disease, myocardial infraction, stroke, amputation due to diabetes-related foot disease, or blindness due to advanced diabetic retinopathy. In addition, hypoglycemia unawareness is a diagnostic criteria for stage 6. Created in Biorender.com.*

with a HbA1c of 9,5% and higher progressed to proliferative retinopathy and macro-albuminuria, respectively, after 20 years (20). Finally, stage 6 T1D would be demarcated by the presence of at least one of the end-stage complications such as end-stage renal disease, myocardial infarction, cerebrovascular accident, amputation due to diabetes-related foot disease, or blindness due to advanced diabetic retinopathy (**Table 1**). Besides these vascular complications, patients with hypoglycemia unawareness should be included in this stage, as it is a risk factor for potentially fatal severe hypoglycemia episodes and its incidence increases with duration of T1D (22). Hypoglycemia unawareness is currently one of the eligibility criteria for islet cell transplantation (23). In line with this, stage 6 signifies the further loss of β cell function and urgent need for β cell replacement therapy as a last resort. Up to 5% of T1D patients will have arrived at stage 6 20 years after the onset of stage 3 (20, 24, 25). Contrastingly, as an illustration of patient heterogeneity, approximately half of T1D Medalists (more than 50 years after stage 3) have yet to reach stage 5 (26), though it should be noted that these patients are the exception to the rule (**Figure 2**). More commonly, T1D patients progress through the stages and the majority of mortality is, consequently, caused by T1D related complications, as was shown in a cohort of childhood-diagnosed T1D in the United States. Approximately 90% of mortality was caused by diabetes-associated complications 30 years after the start of stage 3, by which time around 20% of T1D patients had passed away (27, 28). Together, these examples illustrate that progression through the stages can be diverse. The course of T1D might eventually be better coined as rapidly or slowly progressive, in the same vein as how multiple sclerosis is subdivided in different types. In

this way, the new staging system could therefore not only be a better tool to systematize the therapy need related to the different stages of T1D, but also help to discern different time-courses of progression. In the future, these clinical outcomes could be aligned with auto-immune signatures and β cell function, once these tests are widely available. Patients that progress rapidly would likely have less residual β cell function and higher activity of autoimmunity.

Overall, the intent of re-staging T1D is to reinstall the urgency for T1D treatment discovery and implementation beyond insulin replacement therapy, as it becomes clear that insulin, started at stage 3, does not prevent progression towards stage 6 T1D. Hereby, insulin as a sole treatment is dismissed while more incentive is given towards treatments that prevent or delay progression to irreversible end-organ damage (stage 6). This new staging system also encourages the translation of drugs so far most used in T2D to optimize glucose control in stages 3 to 6 T1D and with this minimize complications and revitalize β cells. At the moment, there is still a stigma related to T1D complications, which are often thought of as being the T1D patient's own responsibility (29). Hopefully, this new staging system will stress that progression through the stages is actually the natural course of T1D which even optimal patient effort to manage glycemic control cannot fully prevent. Hence, a new drug is needed to stop this progression.

2. Balancing the risks and benefits of therapy

Chapter 2 shows that reversing T1D is conceptually possible if defined as being insulin independent. After autologous hematopoietic stem cell transplantation (aHSCT), all patients were insulin-free for a sustained period of time with the exception of three patients who had inadvertently received corticosteroids or had developed diabetic ketoacidosis before the therapy (30, 31). The pursuit of insulin independence came at a risk, however. Even though morbidity and mortality after aHSCT has improved to an incidence of <1% over the years, there is still a chance of serious, life-threatening complications (32). When considering new therapies, one tends to forget that T1D is still a deadly disease, with more than 70% of mortality in T1D patients in the first 10 years attributable to acute consequences of hypoglycemia or diabetic ketoacidosis and after 20 years of diagnosis due to micro- and macrovascular complications (27, 33). Yet, the risk of aHSCT has been valued to be unacceptable and therefore this therapy has not gained much interest in the T1D field. In children with acute lymphoblastic leukemia, on the other hand, allogeneic HSCT is the golden standard for therapy with a 90% 5-year survival rate (34). In this sense, there is a need for more debate on the risks that are imposed by T1D itself versus those of an effective therapy. For each patient, the risks of infrequent but acute and sometimes severe therapy-mediated morbidity would need to be weighed against the so far largely unavoidable and higher eventual T1D-related morbidity.

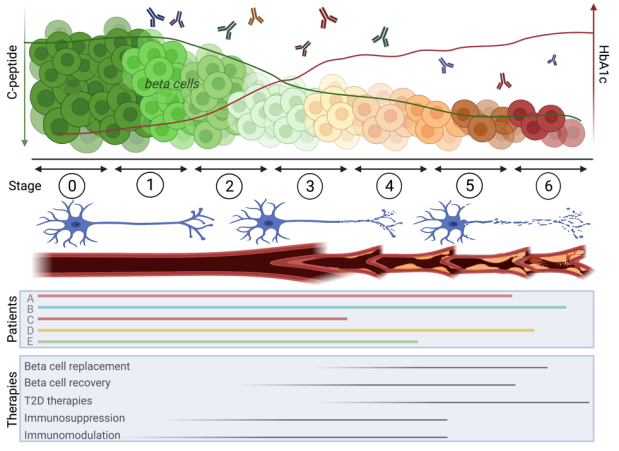


Figure 2: Case Example aided Overview of the Revised Stages of Type 1 Diabetes. *The progression of stages in type 1 diabetes is exemplified by a loss of functional beta cell mass. This is depicted in the top of the figure by a reduction in beta cell mass, a deterioration in function (green are functional beta cells and red are dysfunctional beta cells), an increase in HbA1c, and a reduction in secreted c-peptide. Type 1 diabetes is firstly diagnosed by the detection of two or more autoantibodies in stage 1. In Stage 2 dysglycemia is added, as shown by the increase in HbA1c. After stage 3 (the conventional T1D diagnosis), micro- and macrovascular complications prompt the diagnosis of stage 4 to 6 T1D, as depicted by the deteriorated blood vessels and neurons due to the progression of the disease. Under the graph, a bar graph is shown, in which every bar represents the lifespan of one patient, as an illustration of patient heterogeneity. The length of the bar corresponds to the stage of T1D and the colour suggests slowly- (green) to rapidly- (red) progressive disease. Patient A is a 40-year-old male T1D patient, who was diagnosed with stage 3 T1D at age 9 and quickly progressed to stage 6, ultimately dying of a heart attack. Patient B is a slow-progressor, being diagnosed with stage 3 T1D at age 21 and dying at age 80 due to end-stage renal disease. Patient C is another fast-progressor that sadly died at an age of 16 just after stage 3 T1D diagnosis as a consequence of diabetic ketoacidosis. Patient D diagnosed at 13 years of age with stage 3 T1D steadily progressed through the stages and died of a hospital-acquired pneumonia, when she was hospitalized for a foot amputation at age 69. Finally, patient E is a 72-year-old T1D Medalist only suffering from non-proliferative retinopathy and dying 52 years past stage 3 T1D diagnosis of a T1D-unrelated cause. Under the patient bar graph, another bar graph is shown depicting different T1D therapies. The location and length of the bars align with the stages that would be optimal for implementation of these therapies. Created in Biorender.com.*

Evidently, lowering the risks of T1D therapies like aHSCT will result in an easier choice. In addition, better patient-specific predictors for both disease course and treatment associated morbidity would make these decisions more manageable (**Chapter 2**). For example, all aHSCT patients had some period of insulin free survival, but patients with low baseline autoreactive islet-specific T-cells clearly had more benefit than patients with high frequencies of these cells (31, 35). More intensive analysis of characteristics of responders versus non-responders could give us insight into the predictors for treatment effect or

vice versa risk factors for failure. This will eventually enable risk factor-based selection of patients for a specific therapy. Evidently, this becomes increasingly important as the therapy becomes more toxic.

3. Patient heterogeneity in therapeutic response

Patient heterogeneity was previously touched upon with regard to risk assessment. The higher the risk of a treatment, the more important it becomes to select the right patient population that would benefit from the treatment. aHSCT is undoubtedly an example of that (**Chapter 2**). Patient heterogeneity is, however, a crucial point for T1D therapies in general. The extent of this heterogeneity has become clear only in recent years. The variable therapy success rates observed in subgroups of patients could be argued to reflect the heterogeneity seen in the pathophysiology of patients. Recently, this has been coined as the different 'endotypes' of T1D (36). Endotypes could be based on different T1D characteristics, such as age of onset, HLA-type, autoantibody response and response to therapy. Overall, this means that many therapies previously determined to be ineffective might indeed have had efficacy in certain subgroups of patients unidentified at that point of time, but failed to show an effect in the total study population (37). In this way, we might have inadvertently dismissed many drugs with T1D endotype specific effectiveness.

One other example of patient heterogeneity is our case of remitting T1D after intravenous immunoglobulin (IVIG) treatment (**Chapter 3**). IVIG has been investigated in a randomized controlled trial as a therapy to treat T1D in adults and children without success (38). Yet, one of our patients repeatedly experienced resolution of her T1D after IVIG treatment, exemplified by periods of insulin independence. This summons up the question whether there are more patients similar to her that could benefit from this treatment but have not yet been identified. Indeed, some smaller, older studies did find a decrease in insulin requirements after IVIG treatment in children and newly diagnosed T1D patients (39, 40). It should be noted, however, that our patient was unique with regards to several comorbidities such as chronic inflammatory demyelinating polyneuropathy (CIDP) (which was the indication of IVIG therapy) and Graves's disease, which would likely exclude her from participation in most immune intervention trials. Perhaps the reason she did respond to this treatment, though, is because her endotype is more auto-antibody driven than the 'typical' T1D patient, as she has auto-antibody driven inflammatory comorbidities. One case report showed that a child with T1D and high titers of insulin antibodies had improved glucose control after IVIG treatment, although insulin dose did not decrease (41). Reduction in autoantibody titers after IVIG was replicated by another independent case study (42). In general, the design of current trials still tends to focus on drug effectivity in more homogeneous cohorts while analysis of rare responder (e.g. with

additional comorbidities) instead gives more insight and credit to possible endotype specific effectivity of new therapies.

In summary, instead of posing a challenge, embracing patient and endotype heterogeneity in designing trials could be the savior of T1D therapies.

4. Identification of the T1D patient for preventative strategies

With the advent of the improved diagnostic criteria for T1D and new staging models, T1D patients now include persons with autoantibodies with and without dysglycemia before the conventional T1D diagnosis (15). This model was brought into being to facilitate earlier treatment of T1D in clinical trials. This is exemplified by the success of the teplimuzimab trial, which studied stage 2 T1D patients and showed the delayed onset of stage 3 T1D (43). A caveat to this staging model is that up to 20% of T1D patients test autoantibody negative at diagnosis, though more than half will seroconvert in subsequent years (44, 45). It is yet unclear whether these patients did have detectable autoantibodies at some point before the start of stage 3. Inclusion of pre-stage 3 T1D could have improved the outcomes of trials such as autologous hematopoietic stem cell transplantation that was most successful in patients treated shortly after diagnosis and with sufficient β-cell function (**Chapter 2)**. Identification of stage 1 and 2 T1D patients forms a challenge, however, as these stages are asymptomatic and requires population-based screening. In addition, screening without pre-selection is costly and difficult to defend unless the importance of this approach is justified by shown improved treatment efficacies over the traditionally studied stage 3 T1D group. On the other hand, screening could prevent hospital admissions and thereby even lower eventual health care costs. Indeed, the Fr1da study showed that autoantibody screening of the general population reduced diabetic ketoacidosis at clinical diagnosis of T1D from 16-58% to 3.2% (46-49). Currently, screening is performed by autoantibody detection, but could be preceded by pre-selection on family history; however, only approximately 15% of new cases have a family history of T1D (50). Finally, screening could be guided by HLA genotypes, as 95% of Caucasian T1D patients have the highest HLA-risk haplotype DR3/4 (51, 52). Of note, children with high genetic risk of developing T1D had detectable autoantibodies within the first three years of life so screening should start as early as three years of age (53-55). For such HLA dependent (pre-) screening, HLA typing should be generally available e.g., as part of heel-prick program for newborns.

In conclusion, screening programs could identify T1D patients when they still have sufficient β cell mass and before they necessitate insulin treatment. This facilitates timely enrollment in clinical trials to counter the harmful autoimmunity leading to stage 3 T1D and, concomitantly, reduce possibly fatal early complications of the disease by educating patients and caretakers (56).

5. The goal of T1D treatment

The final challenge in realizing a therapy for T1D is re-considering the goal of a treatment. A cure for T1D is obviously the ultimate goal, but this should not stand in the way of accepting therapies that would halt disease progression or prevent complications and thereby increase quality of life. Therapies that minimize symptoms and reduce the risk of complications are currently also the only immunotherapies on the market for other autoimmune diseases. T1D should not be an exception. Until there is a cure, such disease-modifying therapies could be of immense value to T1D patients. Thus, therapies that could slow the decline of c-peptide secretion by one or many years should not be withheld from T1D patients, especially since we now know that even barely detectable c-peptide levels show a clearly reduced risk of complications and less hypoglycaemic episodes in the disease course (57-60). Therefore, treating T1D rather than curing it, by chronic or intermittent therapy, should be offered.

To conclude, while we are working on finding a cure for autoimmune disease, the goal of T1D treatments at present should be to minimize progression and long-term complications rather than fast-forwarding to a cure only.

Next generation type 1 diabetes therapies

The next generation of T1D therapies would ideally take the previous points into account (**Figure 3**). Firstly, identification of different stages of T1D should guide our choices for trial inclusion and eventual treatment selection, as will be discussed in the forthcoming paragraph (**Figure 2**). In addition, the impact on complications and quality of life will be an important factor influencing which therapy is worth pursuing. Furthermore, a combination of different therapies is likely needed in order to have clinical success (61, 62). Caution should be taken, however, as combining therapies that target multiple immune pathways might pose a risk of inadvertent immunosuppression (61).

Personalized Medicine

Depending on the stage of T1D, various combinations of therapies could be suggested. In the earlier stages (1-2) with still sufficient β cell mass, the emphasis could lie on the anti-inflammatory response, whereas in later stages (3-6) β cell revival or replacement therapies could become more critical (**Figure 2**). Other important factors, besides the patient's stage of T1D need to be taken into account. For instance, patients diagnosed in childhood display a more aggressive immunophenotype than patients who are diagnosed in adulthood and are less likely to have remaining c-peptide secretion a decade after clinical diagnosis (63, 64). Thus, children in stage 1 or 2 might benefit from more aggressive immunosuppression, whereas adults in the same stage could suffice with a

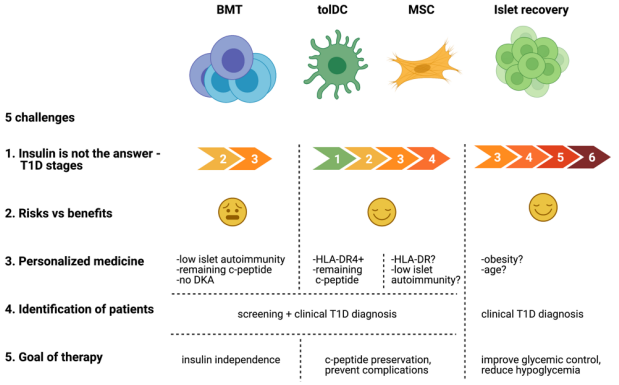


Figure 3: The Five Challenges of Implementing Type 1 Diabetes Therapies. *The preceding chapters have discussed five challenges of implementing T1D therapies. The table above summarizes each challenge as it applies to different therapies. The first column depicts autologous bone marrow transplantation (BMT), the second tolerogenic dendritic cells (tolDCs), the third mesenchymal stromal cells (MSC), and followed lastly by islet recovery therapies. In the first row, the T1D stages are depicted as was shown in Table 1. In row two, the risks versus benefits are illustrated by emoticons. The anxious emoticon in the first column represents a situation in which the risks might outweigh the benefits, whereas the smiley emoticon represents a situation in which the benefits likely outweigh the risks. Created in Biorender.com.*

milder immune inhibiting therapy. Besides the stage and age of the patient, one could select a therapy on the detection of certain autoantibodies, T-cell autoantigen reactivity, and HLA-type. To illustrate, preliminary data from the GAD-alum study suggested that its efficacy was dependent on HLA type (65). Besides, there have been indications that certain antigen therapies were more effective when patients had higher autoantibodies against the tested antigen at baseline. For instance, T1D development was delayed by oral insulin in a subgroup of patients with high insulin autoantibodies (66).

To summarize, it is important to capture the target population in a clinical trial by designing appropriate inclusion and exclusion criteria. The era of treating T1D as a singular disease is past. Patient heterogeneity needs to be embraced to unveil the potentially variously different successful treatments for different T1D endotypes.

Cellular Immunotherapies

Cell therapies are almost by definition personalized. As the majority of this thesis includes cellular immunotherapies, these will now be discussed in more detail. Tolerogenic dendritic cells and mesenchymal stromal cells are of interest as they by themselves

already combine two treatment modalities, namely as antigen-presenting cells but additionally as cells with general anti-inflammatory properties (**Chapter 4 & 5**). Conceivably, antigen-specific therapy would be most beneficial when started earlier in the disease process (stages 1 or 2) before considerable antigen spread occurs (**Figure 3**). This is substantiated by the GAD-alum trial, which showed a slowed decrease in fasting c-peptide four years after cessation of therapy only in T1D patients in the first 6 months of diagnosis, but not in longer standing T1D (67). This suggests that earlier enrollment (stages 1 or 2) could result in improved outcomes.

Tolerogenic Dendritic Cells

Thus far, tolerogenic dendritic cells were shown to be safe and feasible in a phase one trial in primarily stage 4 T1D patients (68). Currently a phase two trial is being planned which will include T1D patients with remaining c-peptide secretion, mimicking a pre-stage 3 situation. Indeed, the best target population for tolDC therapy will likely be stage 1 or 2 T1D patients and stage 3 or 4 T1D with remaining c-peptide secretion (**Figure 3**). TolDC therapy could be followed by anti-CD3 antibody therapy in patients with high auto-autoimmune signatures after a sufficient amount of time, as to not intervene with the beneficial effect of tolDC therapy on Tregs.

Important and indispensable for clinical translation, tolerogenic dendritic cells proved to be stable cellular products in terms of their phenotype and function (**Chapter 4**). The clinical background of the donor, either healthy or with type 1 diabetes, did not change the phenotype, transcriptome, or methylome of tolDCs. Furthermore, mature tolDCs remarkably resembled immature tolDCs with regards to their epigenetic profile, substantiating the claim that tolDCs are locked in a semi-mature state. As methylation is seen as a stability marker, our findings provide confidence that the use of these tolDCs as a cellular therapy constitutes a low risk of their conversion into an inflammatory phenotype. Besides immune-related genes, several T1D risk genes showed to be changed in vitamin D3-(VD3) treated tolDCs when compared to inflammatory DCs, both on a transcriptional and epigenetic level. This could give insights why VD3 supplementation early in life was shown to decrease the chance of developing T1D, as it might offset the T1D genetic risk profile (69, 70). Vitamin D is a pleiotropic hormone, having roles in calcium homeostasis, bone metabolism, and immunity. Immune cells, especially DCs, express VD receptors and the enzyme 1α-hydroxylase that converts vitamin D to its active form VD3, signifying an important role of VD3 in DCs specifically (71). As it is known that VD3 is decreased in T1D patients (72), tolDC therapy may be seen as a specific supplementation of VD3 to one of the cells it acts upon.

Many of the advantages of using tolDCs as a cellular therapy could simultaneously be seen as disadvantages and thus are two sides of the same coin. Several of these need to be discussed.

Firstly, tolDCs pulsed with a diabetogenic peptide is an antigen-specific therapy. In this way, the therapy provides a more targeted immune modulation compared to classical more general immune suppression. On the one hand, this reduces the risk of infection and of cancer compared to general immune suppression, but on the other hand, this targeted immune intervention might not be effective enough to counter the multi auto-antigen directed auto-immune process leading to T1D. Consequently, tolDCs might have to be combined with other immunomodulatory therapies, as proposed previously, for instance, with concomitant Treg infusion (73). The functionality of tolDCs, however, might be altered if produced after the administration of another immunosuppressive therapy. Furthermore, the C19-A3 peptide with which we pulsed tolDCs is a peptide epitope of proinsulin that was found to be well-tolerated and safe, both as a peptide therapy and when presented by tolDCs (68, 74, 75). It has currently only been tested in HLA-DR4 patients, however, thus limiting the number of patients that could benefit from this therapy. Therefore, other peptides need to be examined in order to broaden the patient population eligible for such therapies. Any autoantigen should always be tested with caution, as it might result in antigen-dependent immune activation, but especially these with post-translational modifications, such as defective ribosomal products and hybrid insulin peptides (76, 77). Indeed, treatment of multiple sclerosis (MS) patients with a myelin altered peptide ligand caused exacerbation of MS (78).

Secondly, tolDCs are the patient's own cells, which is advantageous as there is no risk of rejection. However, the functionality of these cells could be affected by suboptimal glycemic control. Our tolDCs from T1D patients did not differ from tolDCs from healthy controls, but our T1D patients were selected to have an HbA1c of less than 8%. A different group, on the other hand, found that tolDCs produced from T1D patients with poor glycemic control (mean HbA1c 10.2%) were less tolerogenic, albeit their tolDCs were produced with vitamin D2 as opposed to VD3 (79, 80). If these results would hold true for our tolDCs, this would limit the target group of tolDCs to patients that are successful in managing their blood glucose, which is approximately 30% of adult T1D patients in the United States and less in adolescents and children (https://t1dexchange.org/). This problem would be solved, however, if indeed the target population would be patients with stage 1 or 2 T1D, as by definition these patients would have a HbA1c of < 7%.

A more relative disadvantage of tolDCs is that patients need to undergo a lengthy leukapheresis process (3-4 hours) in order to retrieve blood cells needed for production of this cellular therapy, which changed the T-cell responses requiring some cases several

months to recover to baseline levels (68). The complete production procedure does not only hamper a speedy implementation of therapy, but also necessitates a laboratory that is equipped and specialized to GMP produce such cells. However, the logistic and clinical burden of tolDCs shows to be moderate, at most, compared to other immunotherapies, such as anti-CD3, which requires at least one set of two weeks of intravenous treatment (43, 81). Finally, an inherent problem with cellular therapies is that the cells have a limited life span and likely also a time limited effect necessitating repeated administration. TolDCs, however, have shown to confer a legacy effect by infectious tolerance and linked suppression, thereby possibly circumventing this problem (82).

In conclusion, tolerogenic dendritic cells are attractive as antigen-specific therapy and have proven to be safe and feasible in T1D patients. Next, a phase two clinical trial should investigate whether C19-A3 pulsed tolDCs are also effective in preserving c-peptide. In the future, other peptides should be tested, in addition to combinations with other immunotherapies, with the best effectivity expected in early stage T1D patients.

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) can be used as an antigen-specific therapy, similarly to tolDCs (**Chapter 5**). We showed that MSCs can be safely activated by pro-inflammatory cytokines to express HLA class II and thereby present antigens (**Figure 3**). This is advantageous as MSCs were already investigated as a treatment for T1D with regards to their combined anti-inflammatory and regenerative potential. MSC therapy preserved c-peptide secretion in recent-onset T1D patients (83). Furthermore, MSCs could be used as an off-the-shelf allogeneic therapy, as risk of rejection is limited because of their hypo-immunogenic nature (84). This facilitates quicker usage, although batch-to-batch variability could be problematic and universal quality control criteria should be implemented.

Similar to tolDCs, disadvantages of MSCs are their costs and the complicated logistics of production and administration. Therapies with living cells in this respect remain intrinsically variable and have to be produced by trained personnel and used in a timely fashion. In the foreseeable future this intrinsic complexity will limit their implementation to the more developed countries. Alternatives for cellular therapies could be nanoparticles or extracellular vesicles. These have less variability as they are not complete cells, but can still relay antigen-specificity (85, 86).

Islets as target of type 1 diabetes therapies

The final report in this thesis touches upon the importance of also engaging the islets of Langerhans in our efforts to treat T1D (**Chapter 6**) (**Figure 3**). Mesenchymal stromal cells can be used simultaneously as immune- and islets supporting agents. In the context of

islet transplantation, these could reduce the amount of islet transplantations necessary by improving the function of the transplanted islets. Besides improving islets for transplantation, islets within a T1D patient could benefit from MSCs or other β cell therapies as well (83). After all, β cell mass is not always reflected into function, as was seen in our case report of relapsing / remitting T1D (**Chapter 3**). This case of T1D alternated functional β cell sufficiency with phases of insulin dependency. Currently, we merely have measures of functioning β cells, namely c-peptide secretion, but as illustrated by our case report, remaining β cell mass might be sufficient, if insulin secretion can be stimulated again. One study showed that 73% of long-standing T1D patients still secreted low levels of c-peptide after a mixed meal stimulation test (21) and another study showed that 58% of T1D patients had residual β cells at autopsy (87). These patients could in theory benefit from approaches that revive these β cells to produce insulin again (21, 88). Most of these so called β cell recovery strategies at the moment target the GLP-1 pathway. Liraglutide, for instance, is a GLP-1 analogue and was shown to significantly reduce HbA1c rates in T1D patients compared to placebo, when combined with insulin (89-92). In addition, it significantly reduced mean body weight by several kilograms depending on the dose (91). Subgroup analysis showed that patients with residual c-peptide secretion had a better clinical outcome than patients with no c-peptide secretion left, suggesting that T1D with endotypes in stage 3 and further with residual c-peptide secretion that are also overweight would benefit most from this therapy. Once c-peptide levels are no longer detectable, strategies to replace β cells or revive the existing dormant β cells could be advised, though clinical translation of this idea is still challenging (93).

A liaison between islet, immune and stromal cell therapies

This thesis illustrates a journey from general immune suppressive therapies towards more islet-specific immunomodulation in T1D. This journey does not occur on a one-way street, however. The take-home message of this thesis, then, is neither that immune suppression per se is flawed nor that antigen-specific immunomodulation is the sole answer to cure all T1D patients. Rather, optimal therapy might likely be a combination of controlled immune suppression and functional antigen-specific immunomodulation capable to protect β cells. Dissecting the endotypes in T1D will help us guide which end of the therapy spectrum is the best fit for each specific patient. The need and type of islet-targeted therapy will furthermore also be determined by the stage of T1D. Above all, the T1D field will benefit from acknowledging that apart from finding a cure, therapies that successfully halt or slow down T1D progression and minimize its long-term complications are additionally worthy to pursue.

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Chapter 8

Summaries (English & Dutch)

List of Publications

Acknowledgements

Curriculum Vitae

English Summary

Type 1 diabetes (T1D) is an auto-immune disease characterized by a T-cell mediated attack on the β cells in the pancreas. Beta cells produce insulin, which lowers blood glucose levels by facilitating the uptake of glucose by peripheral tissues. Therefore, T1D is diagnosed by high blood glucose levels. Recently, a new staging system was introduced that diagnoses T1D before the clinical manifestation of hyperglycemia (stadium 3). Hereby T1D is diagnosed when patients have two or more autoantibodies (stadium 1) and dysglycemia (stadium 2) to build the stage for late prevention or early intervention before disease onset. What exactly causes the onset of diabetes has not yet been unraveled. The immune system is dysregulated in T1D, which leads to destruction of β cells by autoreactive T-cells that have been activated by antigen-presenting dendritic cells. T-cells consequently activate B-cells, which produce autoantibodies that can help with the diagnosis of T1D. After T1D diagnosis, insulin treatment is started to avoid hyperglycemia, to treat the symptoms but not the cause of T1D.

Except for insulin, there are no approved therapies for T1D. Many immune intervention therapies have been investigated, but with limited success. Mono immunotherapies, which consist of antibodies against a certain immune cell, such as CD3+ T-cells or a cytokine, were often times only effective for a certain period of time and only in subgroups of patients. Antigen-specific therapies hold promise because they can selectively inhibit the immune system without causing general immune suppression. Nevertheless, these early efforts struggled to slow disease progression and only show subgroup effectivity at best. Antigen-specific strategies could be combined with cellular therapies to improve their potency, as is accomplished with tolerogenic dendritic cells. In this thesis, various therapies for T1D have been evaluated to gain insights for future T1D therapies.

Firstly, in **part I** the foundation is set for the notion that T1D could be reversed by immunotherapies. In **chapter 2** of part I, the opportunity of using autologous hematopoietic stem cell transplantation (aHSCT) as a treatment for T1D is discussed. Autologous HSCT is thus far the most effective therapy for T1D, resulting in insulin independence in 21 out of 25 patients for a median of 43 months. The success of this therapy is, however, hampered by the possible risks, although mortality has decreased to <1% in recent years. Post-hoc analyses concluded that patients had a better outcome when they had a low auto-immune signature, no diabetic ketoacidosis, and remaining c-peptide secretion prior to the aHSCT. Therefore, we propose that aHSCT should be considered in patients that meet the above-mentioned characteristics and should be discussed through informed decision-making involving the patient and their caregivers.

Many patients will likely value aHSCT as too risky or invasive, even though this is standard of care as successful cancer therapy. Therefore, another immunotherapy was presented in **chapter 3** that also caused the reversal of T1D. A case report showed that a T1D patient became insulin independent repeatedly for several months after intravenous injection of immunoglobulins (IVIG). This illustrates that T1D patients could restore their endogenous insulin production, even after diagnosis and offers hope for immunotherapies as a treatment for T1D. We further concluded that β cell mass does not equal function, as this patient was able to produce sufficient insulin again resulting in insulin independence with presumably the same (or less) β cell mass as when she required insulin. It is yet unclear why this patient responded to IVIG, whereas a randomized controlled IVIG trial was unsuccessful. Like every patient, this case is unique and presented with multiple co-morbid conditions, such as chronic inflammatory demyelinating polyneuropathy (CIDP) and Graves' disease. Therefore, this study reminds us that personalized medicine is of utmost importance and that we need to keep studying individual cases and subgroups to understand disease and heterogeneity thereof, to then apply or develop a successful, tailored therapy for every patient with T1D.

In conclusion, in part I we present that T1D can be reversed by immunotherapy, even though these specific therapies might not be successful or applicable in all cases. Therefore, alternative, milder, targeted and antigen-specific therapies were studied in **part II**. In **chapter 4**, we investigated the stability and reproducibility of tolerogenic dendritic cells (tolDCS) that, when pulsed with peptide, confer antigen-specific immune suppression. TolDCs were produced by treating monocytes of healthy subjects and T1D patients *ex vivo* with VD3 and dexamethasone. The profile of tolDCs was similar between healthy subjects and T1D patients with regards to transcriptomics, methylomics and function. Besides, there was no difference between two international production sites of tolDCs, paving the way for multicenter and international implementation of tolDC therapy. Furthermore, tolDCs were differentially methylated compared to inflammatory dendritic cells and neither changed their phenotype nor function after stimulation with inflammatory stimuli. Both results indicate a stable function of tolDCs. We conclude that tolDCs are a promising, stable, and reproducible therapy for T1D. Next to tolDCs, mesenchymal stromal cells (MSCs) were investigated as a possible antigen-specific cell therapy in **chapter 5**. MSCs are inherently immunomodulatory and thus perfect candidates to be used as 'adjuvant' in antigen-specific therapy. We showed that MSCs express HLA class II in an inflammatory milieu, which is necessary to present antigen to CD4 T-cells and Tregs. This activation did not cause secretion of pro-inflammatory proteins, but instead led to increased expression of immune inhibitory markers such as PD-L1 and IDO. Antigen-pulsed MSCs inhibited proliferation of antigen-specific T-cells, even when these MSCs were no longer present in the co-culture of T-cells and APC. These

results encourage the use of MSCs as an antigen-specific therapy in addition to their natural immunomodulatory function.

Besides immunomodulatory therapies, it is important to optimize the microenvironment of the β cells in the islets of Langerhans. Therefore, in **part III**, **chapter 6**, we investigated whether MSCs could be isolated from the pancreas and whether these could display properties that could benefit β cell vitality and function. We have successfully isolated MSCs from the conventionally discarded pancreas tissue of pancreatic islet isolation. These MSCs secreted more anti-inflammatory factor (TSG-6) and pro-angiogenic factors (VEGF, IL-6, and Il-8) after activation with TNF-α and DMOG. Consequently, these MSCs could have a beneficial effect on the islets of Langerhans with regards to islet transplantation or *in vivo* in T1D patients.

In the **discussion,** five challenges to finding a successful therapy for T1D are addressed. First of all, insulin should not be perceived as a cure for T1D, as long-term complications cannot be prevented, even with optimal insulin therapy. Therefore, we propose to adjust the current T1D staging system to appreciate the full scope of the disease—including complications—in order to underscore that insulin is insufficient to counteract the disease process. The second challenge is the dilemma between risk and benefit of T1D therapy. This dilemma might be alleviated if good inclusion criteria are used for risky treatments so that only patients with the highest chance of benefit would receive the treatment. This further emphasizes the importance of recognizing heterogeneity between T1D patients and how they respond to therapies, which presents the third challenge. The fourth challenge is to identify asymptomatic T1D patients ('stage' 1 and 2). In the future, autoantibody and genetic screening could identify some of these prodromal T1D patients, which could concomitantly reduce the incidence of diabetic ketoacidosis and prompt early treatment to preserve c-peptide secretion. The last challenge is to expand the goals of T1D therapy from treating the symptoms and finding a direct cure to increasing quality of life. At present, therapies are being dismissed because they do not show a sustained effect on c-peptide, although they do cause a slower decline of c-peptide over a couple of years. These therapies could be worthy to pursue, as we now know that even minimal remaining c-peptide secretion could reduce complications later in life. Eventually, immunotherapies should be combined with therapies that improve β cell vitality to offer a sustained beneficial effect for T1D patients.

Nederlandse Samenvatting

Van onderdrukken naar moduleren van het immuunsysteem in type 1 diabetes patiënten

Type 1 diabetes (T1D) is een auto-immuun ziekte die wordt gekarakteriseerd door een T-cel gemedieerde aanval op de β cellen in de alvleesklier. Beta cellen produceren insuline, wat ervoor zorgt dat het glucose gehalte in het bloed daalt door opname van glucose in weefsels. Vandaar dat T1D gediagnosticeerd wordt op basis van hoge bloedglucose waardes. Recentelijk is er een nieuwe stagering van T1D geïntroduceerd, welke de diagnose vervroegt naar de periode voorafgaand aan de hyperglycemie en klinische presentatie (diagnose) van T1D (stadium 3). Hierbij wordt T1D al gediagnosticeerd als patiënten twee of meer autoantistoffen hebben (stadium 1) of daarbij verstoorde bloedsuikerregulatie (dysglycemie; stadium 2). Wat precies de aanleiding geeft tot het ontwikkelen van T1D is nog onduidelijk. In T1D is het immuunsysteem niet voldoende gereguleerd wat leidt tot destructie van β cellen door autoreactieve T-cellen die zijn geactiveerd door antigeen-presenterende dendritische cellen. T-cellen activeren ook B-lymfocyten, welke vervolgens autoantistoffen uitscheiden die helpen in de diagnose van T1D. Na T1D diagnose wordt insuline gegeven als behandeling tegen de hyperglycemie.

Er zijn geen interventietherapieën goedgekeurd voor T1D, behalve toediening van insuline. Vele immuuntherapieën zijn onderzocht, maar met beperkt succes. Mono immuuntherapieën, wat vaak antistoffen zijn tegen een bepaalde immuun cel (zoals T-cellen) of een cytokine, waren vaak alleen effectief voor een beperkte periode en alleen in subgroepen van patiënten. Antigeen-specifieke therapieën zijn veelbelovend omdat ze het immuunsysteem gericht zouden kunnen reguleren, zonder algehele immuun onderdrukking te veroorzaken. Net als bij de mono immuuntherapieën, konden antigeen-specifieke therapieën de uitscheiding van een bijproduct van insuline (c-peptide; wat wordt gebruikt als meetwaarde van insuline uitscheiding) in het beste geval alleen in een subgroep behouden, maar er is nog veel ruimte om veranderingen toe te passen in de opzet van deze studies. Antigeen-specifieke therapieën kunnen bijvoorbeeld worden gecombineerd met cellulaire therapieën om hun potentie te verhogen, zoals wordt gedaan bij tolerogene dendritische cellen. Dendritische cellen zijn de docenten van het immuunsysteem; zij leren aan andere afweercellen of zij in actie moeten komen tegen een antigeen of juist het antigeen moeten accepteren als lichaamseigen. In dit proefschrift zijn verschillende therapieën voor T1D onderzocht met het doel om een aanbeveling te doen voor de toekomst van T1D therapieën.

Ten eerste is in **deel I** de basis gelegd voor de uitspraak dat T1D teruggedraaid kan worden door immuuntherapie. In **hoofdstuk 2** van deel I wordt de mogelijkheid van autologe hematopoiëtische stam cel transplantatie (aHSCT) als therapie voor T1D besproken.

Autologe HSCT betreft stamcellen uit het bloed van de patiënt die een nieuw afweersysteem kunnen vormen. Het is tot dusver de meest effectieve therapie voor T1D met 21 van de 25 patiënten die insuline onafhankelijk werden voor gemiddeld drieënhalf jaar. Het succes van deze therapie wordt echter overschaduwd door de mogelijke risico's; hoewel de kans op overlijden in de laatste jaren is gedaald naar minder dan 1%. Achteraf is gebleken dat patiënten die voorafgaand aan de aHSCT een lager auto-immuun profiel hadden, geen bloedverzuring (diabetische ketoacidose) en resterende c-peptide uitscheiding hadden, een beter resultaat boekten na aHSCT. Wij betogen dat als patiënten geselecteerd worden op deze kenmerken, zij de kans moeten krijgen om in samenspraak met de behandeld arts gebruik makende van *informed decision making* voor aHSCT te kiezen.

Veel patiënten zullen een aHSCT echter als te riskant en ingrijpend achten, vandaar dat in **hoofdstuk 3** een alternatieve immuuntherapie gepresenteerd wordt. Een case report laat zien dat een T1D patiënt bij herhaling insuline onafhankelijk werd voor meerdere maanden na intraveneuze toediening van immunoglobulines (antistoffen) (IVIG). Dit resultaat illustreert dat T1D patiënten, zelfs na diagnose, voldoende werkzame β cellen kunnen hebben voor hun eigen insuline productie en biedt hoop voor immuuntherapieën als behandeling voor T1D. Wij concluderen dat β cel massa niet hetzelfde is als functie, maar ook dat β cellen soms niet meer werken, hoewel ze er nog wel zijn. Deze patiënt was een unieke casus aangezien ze leed aan verschillende andere ziekten zoals, chronische inflammatoire demyeliniserende polyneuropathie (CIDP) en de ziekte van Graves. Deze studie herinnert ons daarom eraan dat persoonsgebonden geneeskunde belangrijk is en dat we naar individu en subgroepen moeten blijven kijken om een succesvolle, op maat gemaakte therapie te kunnen vinden voor elke patiënt met T1D.

Samengevat hebben we in deel I laten zien dat T1D teruggedraaid kan worden door middel van immuuntherapie, maar dat die specifieke therapieën niet succesvol of toepasbaar zullen zijn in alle gevallen. Daarom werden andere mildere, gerichte en antigeen-specifieke therapieën bestudeerd in **deel II**. In **hoofdstuk 4** onderzochten wij de stabiliteit en reproduceerbaarheid van tolerogene dendritische cellen (tolDCs), die gepulsed zijn met een antigeen en zo antigeen-specifieke immuun onderdrukking teweegbrengen. TolDCs werden geproduceerd door de monocyten van gezonde vrijwilligers en T1D patiënten buiten het lichaam te behandelen met vitamine D3 en dexamethason. Het profiel van de tolDCs was vergelijkbaar tussen gezonde vrijwilligers en T1D patiënten op mRNA, functie en DNA methylatie niveau. Bovendien was er ook geen statistisch significant verschil geobserveerd tussen twee internationale productie locaties van de tolDCs, wat gunstig is voor de internationale implementatie van tolDCs. Daarnaast hadden tolDCs een andere epigenetisch profiel dan inflammatoire dendritische cellen en veranderden ze niet op fenotype en functie niveau in reactie op ontstekings stimuli. Beide

bevindingen wijzen op een stabiele functie van tolDCs. Concluderend lijken tolDCs een veelbelovende, reproduceerbare en stabiele therapie voor T1D. Naast tolDCs, werden ook mesenchymale stromale cellen (MSCs) onderzocht als antigeen-specifieke therapie in **hoofdstuk 5**. MSCs zijn van nature al immuunmodulerend en weefselherstellend en dus ideale kandidaten om te benutten als antigeen-specifieke therapie. Wij lieten zien dat MSCs geactiveerd met pro-inflammatoire cytokinen eiwitten tot expressie brengen die nodig zijn om het immuunsysteem te activeren. Deze activatie leidde niet tot de aanmaak van ontstekingsfactoren en MSCs maakten zelfs meer ontstekingsremmende factoren aan, zoals PD-L1 en IDO. Antigeen-gepulsde MSCs konden de groei van T-cellen die reageerden op dat antigeen onderdrukken, zelfs als de MSCs niet meer aanwezig waren in de co-culture van afweercellen. Deze resultaten ondersteunen het gebruik van MSCs als antigeen-specifieke immuunmodulerende therapie.

Naast immuunmodulerende therapieën is het belangrijk om een zo optimaal mogelijke omgeving te creëren voor de β cellen in de eilandjes van Langerhans. Daarom onderzochten wij in **deel III**, **hoofdstuk 6**, of MSCs ook verkregen konden worden uit de pancreas en of zij de microenvironment van β cellen zouden kunnen verbeteren. Uit het weefsel dat normaal wordt weggegooid tijdens het isoleren van eilandjes van Langerhans voor transplantatie, hebben wij succesvol MSCs geïsoleerd. MSCs scheidden meer ontstekingsremmende factor (TSG-6) en factoren die bloedvaten vormen (VEGF, Il-6, and Il-8) uit na activatie met TNF-alpha en DMOG. Deze anti-inflammatoire en angiogene factoren zouden een gunstig effect kunnen hebben op donor eilandjes na transplantatie, of in de alvleesklier van T1D patiënten zelf.

In de **discussie** worden vijf uitdagingen voor het vinden van een succesvolle therapie voor T1D besproken. Ten eerste, zal er geaccepteerd moeten worden dat insuline therapie niet de uiteindelijke oplossing is, aangezien zelfs met optimale insuline therapie complicaties niet geheel voorkomen kunnen worden op lange termijn. Daarom stel ik een nieuwe T1D stagering voor waarin het gehele ziekteproces van T1D wordt gewaardeerd en complicaties worden gecategoriseerd om te illustreren dat insuline onvoldoende is om dit ziekteproces tegen te gaan. De tweede uitdaging is het dilemma tussen risico en voordeel van een therapie voor T1D. Dit dilemma zal verlicht worden als er goede selectiecriteria worden opgesteld voor risicovolle therapieën, waardoor alleen patiënten de therapie krijgen die de hoogste kans hebben op voordeel. Dit benadrukt meteen het belang van het onderkennen van diversiteit van de ziekte en verschillen tussen T1D patiënten, en hoe ze op therapieën reageren, wat de derde uitdaging is. Ten vierde, is de identificatie van asymptomatische T1D patiënten een uitdaging (stadium 1 en 2). Door autoantistof en genetische screening zouden sommige T1D patiënten in de toekomst vroeg geïdentificeerd kunnen worden, waardoor vroege complicaties zoals diabetische ketoacidose voorkomen kunnen worden en alvast een therapie gestart kan worden om c-

peptide secretie te behouden. De laatste uitdaging is dat we het doel van T1D therapie bij moeten stellen van directe genezing naar verbetering van kwaliteit van leven. Op dit moment worden therapieën afgekeurd omdat ze niet een langdurig effect hebben, ook al zorgen ze wel voor een langzamere c-peptide daling voor enkele jaren. Deze therapieën kunnen waardevol zijn, aangezien we nu weten dat zelfs minimale eigen insuline productie complicaties later in het leven kan verminderen. Uiteindelijk moeten immuuntherapieën gecombineerd worden met therapieën die gericht zijn op de β cellen om een langdurig effect te hebben in T1D patiënten.

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Curriculum Vitae

Kayleigh Montana van Megen was born February 20, 1991, in Amsterdam. She received her Gymnasium diploma from the Montessori Lyceum Amsterdam in 2009 and then began her Bachelor's studies at Amsterdam University College, where she pursued the pre-medical track. Additionally, she enjoyed a variety of disciplines such as psychology, art history, and literature. She studied a semester at the University of Melbourne, where she specialized in biomedical sciences with a focus on laboratory research. Her Bachelor's thesis researched the effects of serotonin in the nucleus accumbens shell on glucose concentrations in blood and was conducted at the Amsterdam University Medical Center. This work was awarded the Thesis of Highest Distinction in the Science Faculty. In 2012 she graduated *cum laude* and started the Zigma medical degree ('*zij-instroom geneeskunde*') at the Vrije Universiteit Amsterdam, during which she completed several medical rotations at the University of Stellenbosch in South Africa. This accelerated medical degree (4 years) focused on research by offering additional research-oriented courses and an extended scientific internship of 6 months. Kayleigh successfully completed this internship in the laboratory of Professor Bart Roep at Leiden University Medical Center, which sparked her interest in pursuing a PhD with Prof. Roep. Immediately following completion of her medical degree in 2016, she moved to Los Angeles, California, where she spent 3 years researching immunotherapies in type 1 diabetes. After several medical missions in Peru and Panama, Kayleigh landed back in the Netherlands in 2020, where she worked for a year as a surgical resident (ANIOS), as well as a COVID-19 vaccination doctor. Kayleigh lives on a boat in Amsterdam with her husband and enjoys painting, slacklining, and acroyoga.