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Regulatory T cells in type 1 diabetes: the role of IL-35 in counteracting the disease

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Abstract

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Type 1 diabetes (T1D) is etiologically considered as an autoimmune disease, where insulin-producing β-cells are damaged by autoimmune attacks. Regulatory T (Treg) cells are immune homeostasis cells. In the present thesis I aimed to investigate the role of Treg cells and other immune cells in the early development of T1D. In order to do that, we first determined which immune cells that are altered at an early stage of the T1D development. We found that dendritic cells and plasmacytoid dendritic cells induce the initial immune response.

Next, we investigated the role of Treg cells in multiple low dose streptozotocin (MLDSTZ) induced T1D and in NOD mice. We found that the numbers of Treg cells were increased in both MLDSTZ and NOD mice when the MLDSTZ mice were hyperglycemic. However, the increased Treg cells showed a decreased production of anti-inflammatory cytokines (IL-10, IL-35 and TGF-β) and an increased expression of pro-inflammatory cytokines (IFN-γ and IL-17a). These results revealed that Treg cells switch their phenotype under T1D conditions.

IL-35 administration effectively prevented the development of, and reversed established MLDSTZ induced T1D. Treg cells from IL-35 treated mice showed an increased expression of the Eos transcription factor, accompanied by an increased expression of IL-35 and a decreased expression of IFN-γ and IL-17a. These data indicate that IL-35 administration counteracted the early development of T1D by maintaining the phenotype of the Treg cells. Furthermore, IL-35 administration reversed established T1D in the NOD mouse model by maintaining the phenotype of Treg cells, seemingly by inducing the expression of Eos. Moreover, the circulating level of IL-35 was significantly lowered in both new onset and long-standing T1D patients compared to healthy controls. In addition, patients with T1D with remaining C-peptide had significantly higher levels of IL-35 than patients lacking C-peptide, suggesting that IL-35 might prevent the loss of β-cell mass. In line with this hypothesis, we found that LADA patients had a higher proportion of IL-35$^+$ tolerogenic antigen presenting cells than T1D patients.

Subsequently, we determined the proportions of IL-35$^+$ Treg cells and IL-17a$^+$ Treg cells in T1D patients with diabetic nephropathy (DN), which were age, sex and BMI matched with healthy controls and T1D patients. The proportion of IL-35$^+$ Treg cells was decreased in DN and T1D patients, but IL-17a$^+$ Treg cells were more abundant than in healthy controls. Furthermore, we found that the number of Foxp3$^+$ Treg cells was increased in the kidneys of MLDSTZ mice. However, infiltration of mononuclear cells was seen in kidneys of these mice. In addition, kidney tissues of IL-35 treated MLDSTZ mice did not show any mononuclear cell infiltration. These results demonstrate that IL-35 may be used to prevent mononuclear cell infiltration in kidney diseases.

Our findings indicate that the numbers of Foxp3$^+$ Treg cells are increased in T1D, but that these Treg cells fail to counteract the ongoing immune assault in islets and kidneys of hyperglycemic mice. This could be explained by a phenotypic shift of the Treg cells under hyperglycemic conditions. IL-35 administration reversed established T1D in two different animal models of T1D and prevented mononuclear cell infiltration in the kidneys by maintaining the phenotype of Treg cells.

Keywords: IL-35, regulatory T cells, type 1 diabetes, LADA, kidney and diabetic nephropathy

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"Let us sacrifice our today so that our children can have a better tomorrow."
- A.P.J. Abdul Kalam

Dedicated to my family and friends

मेरे परिवार और दोस्तों को समर्पित

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Soläng C, Luo Z, Mejia-Cordova M, Thorvaldson L, Blixt M, Sandler S[€] and **Singh K**[€#]: Kinetics of immune cell responses in the multiple low dose streptozotocin mouse model of type 1 diabetes. *Manuscript*

II. **Singh K**[#], Kadesjö E, Lindroos J, Hjort M, Lundberg M, Espes D, Carlsson PO, Sandler S[€] and Thorvaldson L[€]: Interleukin-35 administration counteracts established type 1 diabetes – possible involvement of regulatory T cells. *Scientific Reports* 5, 12633; doi: 10.1038/srep12633 (2015).

III. Espes D[*], **Singh K**[*], Sandler and Carlsson PO: Increased interleukin-35 levels in patients with type 1 diabetes with remaining C-peptide. *Diabetes Care*, 2017 Aug;40(8):1090-1095. doi: 10.2337/dc16-2121.

IV. **Singh K**[*#], Martinell M[*], Luo Z, Espes D, Stålhammar J, Sandler S and Carlsson PO: Comparisons of cellular immunology in type 1, type 2 diabetes and LADA patients. *Manuscript*

V. Enström E[*], Luo Z[*], Mejia-Cordova M[*], Varli S[*], Henmalm A, Soläng C, Hjort M, Thorvaldson L, Blixt M, Espes D, Carlsson PO, Hansell P, Sandler S[€] and **Singh K**[€#]: Possible role of regulatory T cells in diabetic nephropathy in type 1 diabetes patients and in murine models of the disease. *Manuscript*

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Additional publications

I. **Singh K**, Sandler S and Espes D: The Increased Circulating Plasma Levels of Vascular Endothelial Growth Factor in Patients with Type 1 Diabetes Do Not Correlate to Metabolic Control. *Journal of Diabetes Research*. 2017;2017:6192896. doi: 10.1155/2017/6192896.

II. Digre A*, **Singh K***, Åbrink M, Reijmers RM, Sandler S, Vlodavsky I, and Li JP: Overexpression of heparanase enhances T lymphocyte activities and intensifies the inflammatory response in a model of murine rheumatoid arthritis. *Scientific Reports* 7, 46299; doi 10.1038/srep46229 (2017). ***Equal contribution.**

III. **Singh K**[#], Hjort M, Thorvaldson L and Sandler S: Concomitant analysis of Helios and Neuropilin-1 as a marker to detect thymic derived regulatory T cells in naïve mice. *Scientific Reports* 5, 7767; doi:10.1038/srep07767 (2015). [#]**Corresponding author**.

IV. Oskarsson ME, **Singh K**, Wang J, Vlodavsky I, Li JP, Westermark GT: Heparan Sulfate proteoglycans are important for islet amyloid formation and islet amyloid polypeptide-induced apoptosis. *Journal of Biological Chemistry*, (2015), 290(24):15121-32.

Contents

Abbreviations

APC	Antigen presenting cell
Breg cell	Regulatory B cell
BMI	Body mass index
CRAMP	Cathelicidin-related antimicrobial peptide
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DN	Diabetic Nephropathy
Foxp3	Forkhead box P3
GLUT	Glucose transporter
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon gamma
IgG	Immunoglobulin
IL	Interleukin
LADA	Latent Autoimmune Diabetes in Adults
MLDSTZ	Multiple low dose streptozotocin
Nrp1	Neuropilin-1
NOD	Non obese diabetic
pDC	Plasmacytoid dendritic cell
PDLN	Pancreatic draining lymph node
pTreg cell	Peripherally induced regulatory T cell
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Tc cell	T killer cell
Th cell	T helper cell
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
Treg cell	Regulatory T cell
tTreg cell	Thymic derived regulatory T cell

Introduction

The cells of our body metabolize glucose from the circulating blood to generate energy. It is vital to maintain the levels of glucose in our body. The hormone insulin plays a pivotal role in maintaining the glucose level. Insulin is a protein produced by the β-cells of the pancreatic islets of Langerhans. A decrease in the insulin concentration or the sensitivity to insulin causes elevated levels of glucose, i.e. hyperglycemia, and ultimately Diabetes Mellitus. Diabetes Mellitus can be further subdivided in several groups; type 1 diabetes (T1D), type 2 diabetes (T2D), latent autoimmune diabetes in the adult (LADA), gestational diabetes mellitus, maturity onset diabetes in the young (MODY) and mitochondrial diabetes[1-12]. In the long term, the diabetic condition can cause several serious complications such as renal failure, neural damage and cardiovascular diseases[13].

The current diagnostic criteria for diabetes are two measurements of the fasting (f) plasma (P)-Glucose >7.0 mmol/L, non-fP-Glucose >12.2 mmol/L, HbA1c (>48 mmol/mol)[14]. However, the clinical presentation and progression of diabetes differs widely. Earlier, diabetes was classified into juvenile or mature onset diabetes based on the age at onset. In 1980, the classification of diabetes was made based on the requirement of insulin, i.e. insulin dependent diabetes, which is known as T1D, and non-insulin dependent diabetes or T2D. Later, Tuomi et al defined a new group of diabetes, designated Latent Autoimmune Diabetes in Adult. LADA is based on the following diagnosis criteria age > 35 years, presence of glutamate decarboxylase antibodies (GAD)-65 autoantibodies, and detectable endogenous insulin secretion at least 6 months after diagnosis[15]. These patients are phenotypically indistinguishable from T2D at diagnosis, but after over time the disease become more like T1D.

Today, about 385 million people all over the world are suffering from diabetes. Among this population, approximately 10% are suffering from T1D, and a large proportion of the population belongs to the T2D group[16]. In T1D, hyperglycemia is caused by an insufficient production of insulin due to an immune related destruction of the β-cells. These cells fail to produce enough insulin, since immune cells destroy them by mistake. This destruction could be triggered by genetic factors and/or environmental factors, such as infec-

tions[16]. At the early stage of the disease, infiltrating mononuclear immune cells can be observed in the pancreatic islets, so called "insulitis". However, there has been a debate concerning whether T1D is an autoimmune disease or not. Nevertheless, several reports support the notion that T1D is an autoimmune disease. In line with this, it has been reported that T1D patients who were treated with cyclosporine A (an immunosuppressive drug) required a lower amount of insulin[17], suggesting a role of immune cells in human T1D. In addition, a recurrence of disease has been reported after pancreas transplantation in patients who were not treated with immunosuppressive drugs after the transplantation because the donor and the recipient were monozygotic twins, suggesting an ongoing autoimmune destruction in the absence of a transplant rejection[18,19]. Moreover, insulitis has been reported in human T1D patients[20,21] and autoreactive $CD8^+$ T cells have been shown in pancreatic tissue of human T1D[22]. Thus, all these reports suggest that T1D is an autoimmune disease.

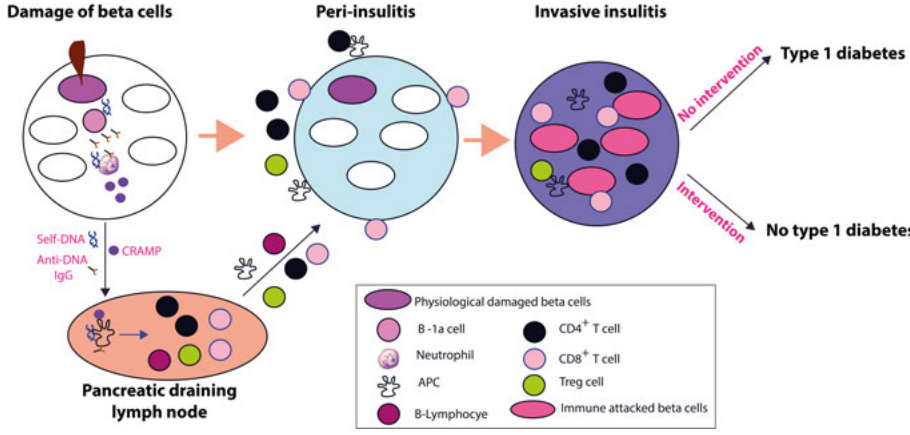

Figure 1. Schematic presentation of the hypothetical immunopathology of type 1 diabetes. Modified from reference 21.

It has been hypothesized that T1D develops when unknown factor(s) damage β-cells (Fig. 1)[23]. The damage causes a release of genetic material[24] from the β-cells. This genetic material could be taken up by innate immune cells, i.e. macrophages, neutrophils, and/or activated B-1a cells. The B-1a cells produce immunoglobulins (IgGs), which trigger neutrophils to release cathelicidin-related antimicrobial peptide (CRAMP) that further binds to DNA. These molecules further activate other immune cells such as antigen presenting cells (APCs), plasmacytoid dendritic cells (pDCs), B-lymphocytes and T-lymphocytes. This phenomenon mainly occurs in pancreatic draining lymph nodes (PDLNs). The activated APCs, B and T cells migrate to the pancreatic islets. Along with all of these immune cells there is

another subset of T cells, which regulate the immune system, known as regulatory T (Treg) cells, and these cells also migrate to the pancreas. Interventions at this stage may halt the disease progress[23,25].

Diabetic nephropathy

Diabetic nephropathy (DN) is one of the most serious diabetic complications and it is the leading cause of premature death and morbidity in diabetes[26]. DN in the clinic is characterized by a reduced glomerular filtration rate, albuminuria and increased serum creatinine[27]. Poor glycemic control and diabetes duration are the major risk factors for the development of DN[28], but they cannot by themselves account for all the pathological changes that occur in the diabetic kidney. Several different mechanisms have been suggested. For example, advanced glycation end products (AGE), activation of protein kinase C and overexpression of certain growth factors have been linked to DN. But perhaps more importantly, studies have suggested that inflammation and immune cells are involved in both the development and the progression of DN[29].

Infiltration of immune cells has been seen in kidney tissues of both animal models and human DN patients. Chow et al have reported earlier that the degree of renal injury was positively correlated with macrophage infiltration in kidney tissues in a T2D mouse model[30]. Furthermore, Ikezumi et al have shown that accumulation of macrophages induces renal injury in a mouse model[31]. In addition, a positive correlation between kidney interstitial $CD4^+$, $CD8^+$ and $CD20^+$ cells and 24-hours proteinuria have been shown in diabetic patients with DN[32]. Eller et al have reported that depletion of Treg cells in a mouse model of T2D increased both insulin sensitivity and nephropathy[33]. Adaptive transfer of $CD4^+Foxp3^+$ Treg cells into diabetic db/db mice significantly improved both insulin sensitivity and nephropathy[33], suggesting a protective role of Treg cells in DN.

Regulatory T cells

The concept of regulatory immune cells was introduced in 1980s, but a lack of phenotypic characterization of these cells hampered this field at that time. In 1995, Sakaguchi and his team reported that cells expressing the markers CD4 and CD25 are immune suppressive cells. However, CD25 was later reported to also function as an activation marker for T cells[34]. This finding questioned whether the $CD4^+CD25^+$ population consists strictly of Treg cells. In 2003, the intracellular transcription factor forkhead box P3 (Foxp3)

was reported to be a lineage marker for immune Treg cells together with CD4 and CD25[35-37]. In the absence of this gene, both humans and mice develop immunological disorders[35-37]. In 2005, Bruder et al. showed that neuropilin-1 (Nrp1) could be used as a surface marker for Treg cells in mice[38], but not in humans[39].

There are two main subsets of Treg cells - the thymic derived, or naturally occurring, Treg cells (tTreg) and the peripherally induced regulatory T (pTreg) cells[40,41]. These cells can be distinguished by using the IKAROS transcription family member Helios as a marker for tTreg cells together with CD4, CD25 and Foxp3[42]. It has also been reported that Nrp1 could be used as a marker for tTreg cells in mice[43,44] but recently, we have shown that Helios is a better marker for tTreg cells than Nrp1 in naïve mice[45].

Treg cells play a pivotal role in maintaining the homeostasis of the immune system by producing granzyme A or B, increasing the consumption of IL-2 to destroy effector T cells by metabolic disruption, and enhancing the DCs to produce indoleamine 2,3-dioxygenase to suppress the effector T cells. They also secrete anti-inflammatory cytokines such as interleukin-10 (IL-10), the newly discovered cytokine IL-35 and transforming growth factor-beta (TGF-β)[46]. It has been reported that cytotoxic T-lymphocyte associated protein 4 (CTLA-4) could also be used as a marker to determine the suppressive activity of Treg cells[46,47].

In T1D patients, the number of Treg cells is debatable since both an increase and a decrease of Treg cells in T1D individuals compared to healthy individuals[48-52]. T1D patients have also been shown to have a defective suppressive function of the Treg cells[53]. Treg cells isolated from the PDLNs of T1D patients have a lower suppressive function compared to healthy individuals[54,55]. Furthermore, it has been reported that in early development of T1D, Treg cells acquire an effector T cell phenotype[56,57]. However, it is not well defined whether or not the tTreg and/or pTreg cells numbers are increased, and which Treg cell subset acquires a T effector phenotype. Also, when, where and why Treg cells switch their phenotype under T1D conditions is not fully understood.

Regulatory B cells

The suppressive function of Breg cells was first claimed in the mid 1970's[58] after experiments with adoptive transfer to guinea pigs, where transferred B-cell depleted spleen cells were unable to suppress delayed-type hypersensitivity. The mechanism, however, could not be revealed at that time and the

field of suppressor B cells was deserted for the next 20 years. In 1996, Wolf *et al.* found that mice with B cell-deficiency could not recover from experimental autoimmune encephalitis (EAE)[59]. Recent evidences support that the suppressive function of Breg cells is due to their IL-10 production[60,61]. Breg cells have been shown to skew T cell differentiation to adopt a more regulatory phenotype[62]. In chimeric mice, a reduction in the IL-10 producing B cells lead to a lower number of Treg cells and instead lead to an accumulation of T helper (Th) cells, Th1 and Th17, in the lymph nodes[62]. Furthermore, in the same study, a longer interaction time between Breg cell and $CD4^+$ T cells were suggested as the mechanism responsible for shifting the phenotype of T cells to a Treg phenotype. Hence, a body of evidence points towards that Breg cells regulate the T cell response and increase the number of Treg cell to modulate the response of the immune system against inflammation. The release of IL-10 is one pivotal regulatory function of Breg cells, together with the secretion of TGF-β and IL-35[63,64].

Despite intensive research in the field of Breg cells and T1D, the role of Breg cells in T1D is not yet clear. Deng et al have reported a lowered frequency of IL-10 producing Breg cells in T1D patients compared to LADA and T2D patients [65], suggesting that T1D patients lose the self-tolerance due to a low number of IL-10 producing Breg cells. Following the same reasoning, Kleffel et al have shown that IL-10 producing Breg cell were more abundant in numbers in long term normoglycemic NOD mice than in hyperglycemic NOD mice[66].

Tolerogenic antigen presenting cells

APCs are mainly studied as potent stimulators of adaptive immunity. However, there is a growing body of evidence suggesting that APCs can also promote, establish and maintain immunological tolerance[67]. Those APCs are called tolerogenic APCs and there are no definitive markers to distinguish these cells from conventional APCs. Indeed, tolerogenic APCs are mainly characterized based on the production of IL-10 and the proliferative induction of both Treg and Breg cells[68-70]. Recently, it has been reported that human tolerogenic APCs produce IL-35[71].

Interleukin-10

IL-10 is an anti-inflammatory cytokine, which could be produced by tolerogenic APCs, regulatory B (Breg) cells, Treg cells and T helper type 2 (Th2) cells. It has been reported that IL-10 prevents the development of T1D in the

NOD mouse model by increasing the number of $CD4^+CD25^+$ Treg cells[72,73]. However, Balaji et al. have shown that IL-10 accelerate the disease in the NOD mouse model in the presence of ICAM-1[74], so the reports so far are conflicting.

Interleukin-35, a novel anti-inflammatory cytokine

In 1997, Devergne et al. showed that the Epstein-Barr virus can heterodimerize with IL-12 alpha (IL-12a or p35)[75]. Furthermore, in 2001, the same group reported that the Ebi3/p35 heterodimer could be immunoprecipitated from the trophoblasts of the human placenta[76]. They further speculated that this heterodimer (Ebi3/p35) play a role in immune regulation[76,77], a notion that was further supported by the research groups of both Vignali and Liew. They reported that the Ebi3/p35 heterodimer cytokine is produce by the Treg cells and that it prevents collagen induced rheumatoid arthritis and colitis in mice[78,79]. Furthermore, Collison et al. found that $Ebi3^{-/-}$ or $IL\text{-}12a^{-/-}$ deficient Treg cells failed to control colitis in mice[79]. However, treatment of recombinant IL-35 in mice enhanced the suppressive function of Treg cells by increasing the number of these cells[79]. In line with this, Bettinni et al. have shown that ectopic expression of IL-35 in β-cells prevents the development of T1D in NOD mice by arresting the proliferation of islet specific $CD4^+$ and $CD8^+$ T cells at the G1:S transition phase[80]. Recently, it has been shown that Breg cells could also produce IL-35[64,81]. It has also been reported that the circulating level of IL-35 is lower in patients with immune thrombocytopenia[82] and active systemic lupus erythematous[83] compared to healthy subjects. Despite these reports, the kinetics of the IL-35 response in autoimmune diseases, such as T1D, is not yet clear.

Transforming growth factor-beta (TGF-β)

The cytokine TGF-β also has anti-inflammatory properties, and it is produced by most tissues under basal conditions, unlike IL-10 and IL-35[84]. TGF-β is required for the development of Treg cells[85]. In addition, it has been reported that the insulin specific Treg cells require TGF-β signaling to dampen the autoimmune response in pancreatic islets[86]. Furthermore, Wallberg et al. have shown a prolonged β-cell survival in NOD mice upon TGF-β overexpression in islets[87]. Although, it has been reported that TGF-β is also required for Th17 cell differentiation[88]. In addition, increased plasma levels of TGF-β have been reported in T1D patients compared to healthy subjects[89].

Proinflammatory cytokines and type 1 diabetes

The production of proinflammatory cytokines such as interferon gamma (IFN-γ), IL-1β, IL-17 and tumor necrosis factor-alpha (TNF-α) are associated with T1D. IFN-γ and TNF-α are considered to be Th1 type cytokines[90]. Several reports have suggested that IFN-γ alone and/or together with IL-1β and/or TNF-α destroy the β-cells of rodents and humans[91-97]. It has also been reported that IFN-γ plays a crucial role in human T1D[90]. Furthermore, a monoclonal antibody to IFN-γ can protect NOD mice and BB rats from the development of T1D[98-100]. Three weeks old NOD mice, which were treated with a monoclonal antibody against TNF-α, did not develop hyperglycemia and did not show any sign of insulitis[101]. In addition, Rydgren et al. have shown a complete protection against IL-1β induced cytotoxicity to rat islets *in vitro* by using an IL-1 trap[102]. The proinflammatory and apoptotic role of the IL-17 cytokine has been shown in human islets[103]. In NOD mice, the inhibition of Th17 cells by neutralizing IL-17 prevented the development of autoimmune diabetes[104,105]. Altogether, the literature suggests that proinflammatory cytokines play a pivotal role during the early development of T1D.

The Ikaros transcription factor family

Transcription factors are called "master regulators" since they regulate the hematopoiesis by controlling the differentiation and proliferation of particular cells[106,107]. The Ikaros transcription family has five members; Ikaros (IKZF1), Aiolos (IKZF3), Helios (IKZF2), Eos (IKZF4) and Pegasus (IKZF5)[107]. All of these members are characterized by two sets of conserved C_2H_2-type zinc finger motifs[108-110].

Ikaros has been reported to be a transcriptional repressor of the IL-2 gene[111] , and Helios regulates the IL-2 gene in Treg cells [112]. Furthermore, it has been reported that Helios could be used as a marker for tTreg cells [42,45]. Nevertheless, over-expression of Helios by T cells could also indicate auto-reactive activities of T cells [113,114]. Eos maintains the phenotype of Treg cells together with Foxp3 [115]. Quintana et al. have shown that Aiolos is required for the Th17 cell differentiation [116]. Hitherto, there has not been shown a direct link of between members of the Ikaros transcription family and T1D.

Aims

The specific aim of this thesis was to elucidate the role of Treg cells in the early development of T1D, and specifically this thesis addresses the following research questions:

1. Which are the immune cells that are altered during the early development of T1D?
2. What is the role of Treg cells during the early development of T1D?
3. Why do the majority of patients with T1D have remaining C-peptide; is there any immunological explanation behind this?
4. What are the cellular immunological differences between patients with LADA, T1D and T2D?
5. What is the role of Treg cells during the early development of diabetic nephropathy?

Materials and methods

Animals

Animals were used in accordance with international guidelines (NIH publications 85–23), and the local animal ethics committee at Uppsala University approved the animal experiments.

Male CD-1 mice were obtained from Charles River (Hannover, Germany). The NOD mice used were originally obtained from the Clea Company (Aobadi, Japan), and have subsequently been bred under pathogen-free conditions at the animal department, Biomedical Center, Uppsala University, Uppsala, Sweden. The NOD mouse strain was derived from the ICMR mouse strain, which is an inbred strain.

The multiple low dose streptozotocin (MLDSTZ) mouse model

Streptozotocin (STZ) is an analogue of glucose[117,118], which has diabetogenic properties in rodents[119-121], dogs and pigs[122,123]. STZ causes DNA damage in insulin producing β-cells[124] and is transported via GLUT2 transporters[124,125]. Five consecutive low doses of STZ trigger an immune response, which lead to β-cell damage and an autoimmune response that results in hyperglycemia[119]. On the other hand, a single high dose of STZ damages the β-cells due to a toxic effect, which leads to hyperglycemia[119,126,127]. The STZ induced immune response is both sex and strain specific[128,129]. In particular, CD-1 and C57BL/Ks male mice develop insulitis[128,129], which is believed to be T cell dependent[130-132].

In the present studies (papers I, II and V) male CD-1 mice were injected intraperitoneally (i.p.) with STZ (Sigma Aldrich), St Louis, MO, USA; 40 mg/kg body weight) dissolved in saline or 200 µl of saline (vehicle) for 5 consecutive days, starting on day 0.

The NOD mouse model of T1D

The NOD mouse model was used as a spontaneous model of T1D in studies II and V. It has been reported that NOD female mice develop hyperglycemia spontaneously, like human T1D patients, and over 20 genetic loci have been shown to be common in NOD mice and human T1D patients[133,134]. Most NOD mice develop hyperglycemia at around 12-14 weeks of age, but a few of the mice can also develop the disease at 24 weeks or later[135].

Blood glucose measurements

Blood glucose concentrations were measured using a blood glucose meter (Medisense, London, UK). Blood samples were obtained from the tail vein of non-fasted mice. Blood glucose levels above 11.1 mM were considered hyperglycemic.

Tissue preparation from animals

The mice were sacrificed by cervical dislocation. PDLNs were removed and placed on ice in RPMI 1640 medium (Sigma Aldrich) supplemented with antibiotics (papers I, II and V). The spleens were also removed and a small piece (approximately 1/3 of the spleen) was fixed in 10% formalin. The remaining spleens were placed on ice in Hanks' balanced salt solution (Sigma Aldrich) supplemented with antibiotics (papers I, II and V). The thymic glands were removed and placed on ice in Hanks' balanced salt solution (papers I and II).

A piece of pancreas (approximately 1/10) was immediately removed, flash-frozen in liquid nitrogen and stored at -80°C until RNA isolation (paper II). The remaining part of the pancreas was transferred to 10% formalin for morphological analysis (paper II). For a few of the experiments in both paper II and V, the whole pancreas was fixed in 10% formalin.

Cell isolation from thymic glands, spleens and PDLNs

Single cell suspensions of thymic glands and spleen tissues were made as follows. The tissues were mechanically disrupted to release the cells. The cell suspensions were centrifuged and the pellets were suspended in 5 ml 0.2 M NH_4Cl for 10 min at room temperature to lyse the erythrocytes. The cell suspensions were washed with Hanks' balanced salt solution and filtered by using a strainer to remove tissue capsules and other debris. PDLNs were

dissected and ground through a mesh to release the cells and then washed with RPMI 1640 medium (Sigma Aldrich).

Flow cytometry staining

Cells were stained for expression of the intracellular transcription factor Foxp3 according to the staining procedure described in the Mouse Regulatory T Cell Staining Kit # 3 (eBioscience, San Diego, CA, USA). In the present studies we did not stimulate and/or used Golgi blocker prior to cytokine staining since it could have caused artifacts upon Foxp3 staining[136]. Further details are provided in the papers concerning the antibodies used in the respective papers.

Sorting of $CD4^+CD25^+$ Treg cells

The single cell suspensions of the thymic glands, spleens and PDLNs were prepared, followed by sorting of the $CD4^+CD25^+$ Treg cells (paper II) by using the Miltenyi Biotec magnetic sorter (Miltenyi Biotec, Germany) and $CD4^+CD25^+$ T cell isolation kit (Miltenyi Biotec), following the manufacturer's instruction.

In vitro stimulation

Sorted $CD4^+CD25^+$ Treg cells (paper II) were cultured in flat bottom plates with RPMI 1640 (Sigma Aldrich) supplemented with antibiotics. The cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) for 1 day or 3 days. In paper II, the supernatants of stimulated $CD4^+CD25^+$ Treg cells were studied to determine the concentrations of IL-10, IL-35 and TGF-β using ELISA kits.

Morphological analysis of pancreatic tissues

Paraffin embedded pancreatic tissues were sectioned at 5-7 μm thickness. In between each section, 5-6 sections were discarded to cover the entire tissue area and to avoid including the same cells in consecutive sections. In total 5 slides containing 4-5 tissue sections on each slide were prepared from each mouse for haematoxylin and eosin staining. The slides were analyzed in a blinded manner under a light microscope as described in papers II and V.

Histological analysis of Foxp3$^+$ cells in pancreas, spleen and kidney

In papers II and V the number of Foxp3$^+$ cells in pancreas, spleen and kidney tissues was determined using immunohistochemistry. We stained formalin fixed tissues with Foxp3 antibodies (eBioscience) and counter stained with haematoxylin. Foxp3 stained tissue sections were analyzed with a DAS Mikroskop LEITZ DMR microscope. The numbers of Foxp3$^+$ cells were ranked according to an arbitrary classification. For further details see papers II and V.

Histological analysis of Ebi3$^+$ cells among Foxp3$^+$ cells in pancreatic tissue

The expression of IL-35 by Foxp3$^+$ cells was determined in the pancreatic tissues. Ten consecutive sections from paraffin embedded pancreatic tissues were made. Among these sections, five alternate sections were stained for Foxp3 and the remaining sections were stained for Ebi3 (a subunit of IL-35) (R&D Systems). Spleen tissue sections were used as positive controls. Consecutive sections stained for Ebi3 or Foxp3 were analyzed using a light microscope. For further details see paper II.

Quantitative RT-PCR

In paper II, total RNA was extracted from PDLNs and spleen cells using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To isolate total RNA from pancreas, the RNeasy Mini kit (Qiagen) was used to improve the yield. cDNA was made from RNA using a reverse transcriptase kit (QuantiTect Reverse Transcription, Qiagen) and random primers supplied by the manufacturer. Real-Time PCR was performed for detection of *Foxp3* in PDLN, spleen and pancreas cDNA, using β-actin as the housekeeping gene.

The information about probes and primers used for Real-Time PCR is provided in paper II.

IL-35 administration

Mouse recombinant IL-35 (Chimerigen, Liestal, Switzerland) was administered i.p. (0.75 μg/day, dissolved in 200 μl PBS) to the mice. The control

group received 200 μl PBS/day and blood glucose concentrations were monitored daily. Four different protocols were employed to study the effects of IL-35 administration. For further details see paper II.

Insulin staining of pancreatic tissues

Pancreatic sections of IL-35 or PBS treated mice were stained for insulin to determine the β-cell number in islets. For further details of staining and analysis, see paper II.

In vitro IL-35 treatment

To elucidate the *in vitro* effect of IL-35 on Treg cells, single cell suspensions of thymic glands, PDLNs and spleens of NOD diabetic mice (> 27.1 mM, blood glucose) were stimulated with plate bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) in 24-well plates overnight in the presence or absence of IL-35 (10 ng/ml). The next day, cells were harvested and stained for flow cytometry analysis.

Enzyme-linked immunosorbent assay (ELISA)

Mouse serum samples were analyzed in duplicate to determine the insulin (Mercodia, Uppsala, Sweden), IL-10 (R&D Systems), IL-35 (Biolegend) and TGF-β (Biolegend) concentrations in mice. The concentrations of IL-10, IL-35 and TGF-β were also determined in cell supernatants of stimulated $CD4^+CD25^+$ Treg cells (50×10^4 cells/well) of diabetic mice by using the same ELISA kits. Plasma IL-35 concentrations in humans were determined by using a IL-35 ELISA kit (Biolegend).

Human whole blood and plasma samples

The analysis of human whole blood and plasma samples was approved by the Uppsala County regional ethics board and carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All participants were supplied with oral and written information and gave written consent prior to inclusion.

Peripheral blood mononuclear cells from freshly isolated whole blood were prepared using Hisopaque-1077 (Sigma Aldrich). The isolated cells were stained for flow cytometry as described above and in papers III and IV.

Statistical analysis

The Sigmaplot 12.03 and GraphPad Prism 6.0 software were used for the statistical analysis. Unpaired t-tests were used for comparisons between two groups. Mann-Whitney Rank Sum Tests were performed for nonparametric observations. One-way ANOVAs followed by Tukey's test were performed for multiple comparisons. The results are expressed as means ± SEM. A p-value below 0.05 was considered statistically significant. Detailed information on what tests were used for the different experiments is included in the figure legends of papers I-V.

Results and discussion

Paper I

T1D is etiologically considered as an autoimmune disease. Despite intensive research in the field of T1D, the kinetics of the innate and adaptive immune cell responses have not been much studied yet. Therefore, in the present study we determined the proportions of innate and adaptive immune cells in thymic glands, PDLNS and spleens of MLDSTZ treated mice. Herein, we used MLDSTZ mice since this model allows to follow the kinetics of disease development closely[119].

The proportions of $CD11b^{-}c^{+}$ DCs were increased in thymic glands on day 3 and in PDLNs on days 3, 10 and 21, and in the spleen on day 10 of MLDSTZ mice after the first injection of STZ. The proportion of pDCs was increased on day 3 in PDLNs and on day 10 in spleens of MLDSTZ treated mice. As for the B-1a lymphocytes proportion, it was increased on day 10 in PDLNs and spleens, and on day 21 in thymic glands of MLDSTZ treated mice. Concerning neutrophils, the proportion was only increased on day 10 in PDLNs of MLDSTZ mice. For Th1 cells, the proportion was increased from day 7 and onwards in spleens and on day 21 in PDLNs of MLDSTZ mice. The proportion of $CD8^{+}$ T cells was increased on day 7 in thymic glands and on days 7 and 10 in PDLNs of MLDSTZ mice. Thus, our data illustrate that the initial immune response during early development of T1D is preceded by DCs and pDCs in MLDSTZ mice.

Paper II

In this study we elucidated the kinetics of the $Foxp3^{+}$ Treg cell response in the early development of T1D. To study this, we used the MLDSTZ mouse model, since the mice in this model develop hyperglycemia and insulitis gradually, which may be similar to human T1D. Herein, we have studied thymic glands, PDLNs and spleens to assess the central, local and systemic immune status. We determined the proportions of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells by using flow cytometry on days 0, 3, 7, 10, 14 and 21 after the first injection of STZ. The $Foxp3^{+}$ Treg cells were increased on day 7 in the thymic glands, from day 7 onwards in PDLNs and on day 21 in the spleen of

MLDSTZ treated mice. The proportions of $Foxp3^+Helios^+$ tTreg cells and $Foxp3^+Helios^-$ pTreg cells were also increased in MLDSTZ treated mice. This data set illustrates that the proportion of $Foxp3^+$ Treg cells is increased in the MLDSTZ induced T1D model compared to controls. However, the upregulation of Treg cells did not counteract the development of hyperglycemia.

We speculated that these results might be explained by the fact that Treg cells may have lost their anti-inflammatory function. To further investigate this, the $CD4^+CD25^+$ Treg cells from MLDSTZ induced diabetic mice and vehicle treated mice were sorted. The sorted $CD4^+CD25^+$ Treg cells were stimulated overnight with plate bound anti-CD3 and CD28, and on the next day the concentrations of IL-10, IL-35 and TGF-β in the supernatants were determined. Interestingly, we found that the concentration of IL-10 was significantly lower in the supernatants of $CD4^+CD25^+$ Treg cells of PDLNs and spleens of MLDSTZ treated mice compared to controls. In addition, the concentration of IL-35 was significantly decreased in the thymic glands and PDLNs of MLDSTZ treated mice. Furthermore, the concentration of TGF-β was also significantly lower in the thymic glands, PDLNs and spleens from MLDSTZ treated mice compared to vehicle treated mice. The relative mRNA expressions of IL-10, IL-35 subunits (Ebi3 and IL-12p35) and TGF-β were impaired in the pancreas (on day 10), PDLNs (on day 10) and spleen (on day 21) of MLDSTZ treated mice. The mean fluorescence intensities (MFIs) of Ebi3 and IL-12p35 were also impaired in the Treg cells from thymic glands, PDLNs and spleens of MLDSTZ treated mice. Altogether, these results suggest that the Treg cells fail in producing anti-inflammatory cytokines in MLDSTZ induced T1D.

Under autoimmune and inflammatory conditions, Treg cells produce more pro-inflammatory cytokines, such as IFN-γ and/or IL-17a, instead of secreting anti-inflammatory cytokines[137]. In our study, we found that the proportions of IFN-γ^+ or IL-$17a^+$ cells among tTreg and pTreg cells were increased in thymic glands, PDLNs and spleens of MLDSTZ treated mice. Thus, this data support our previous hypothesis and reveal that in our model, Treg cells switch their phenotype in MLDSTZ induced T1D. Furthermore, this could be the explanation as to why the increased proportions of Treg cells did not protect against hyperglycemia.

However, it has been reported that phenotypically switched Treg cells could still be suppressive[138]. Therefore, we determined the proportions of $CD4^+CD25^-$ T cells and $CD4^+CD25^-$IL-$17a^+$ (Th17) cells. It is well known that Treg cells suppress the proliferation of $CD4^+CD25^-$ T cells and the differentiation of Th17 cells[46]. In our model we found that $CD4^+CD25^-$ T cells

were initially decreased (on day 7), but increased from day 10 in PDLNs, when the mice had become diabetic. Furthermore, Th17 cells were increased in the PDLNs (from day 10) and the spleens (on day 21) of the MLDSTZ treated mice. These findings suggest that phenotypically shifted Treg cells were not suppressive in our model.

Tang et al. have shown that Treg cells also switch their phenotype in the NOD mouse model of T1D, and that this is due to a defective production of IL-2 by the effector T cells[139]. In addition, Tang et al. also reported that the apoptotic stability of Foxp3 was decreased in the NOD mouse model due to an insufficient IL-2 production[139]. Therefore, we determined the expression of IL-2 in lymphocytes, $CD4^+CD25^-$ and $CD8^+$ T cells by flow cytometry analysis. We also used Bcl-2 to determine the stability of the Foxp3 gene[140]. We found that the production of IL-2 was not insufficient and that the Foxp3 was stable in all the studied organs/tissues. However, the *Foxp3* gene was not stable in PDLNs on day 21.

Pang et al. have reported that Eos together with Foxp3 plays a crucial role in maintaining the phenotype of the Treg cells[115]. Therefore, we examined the expression of Eos in Treg cells from thymic glands, PDLNs and spleens. We found that the Eos expression was decreased in the Treg cells of the thymic glands on day 3, and increased on day 7 (when the mice were not diabetic) in the PDLNs and spleens of MLDSTZ mice. However, the expression of Eos was impaired in all the studied tissues on days 10 and 21, when the mice were diabetic. Thus, our results illustrate that Treg cells switch their phenotype under the T1D conditions in our model, and that this may be caused by an impaired expression of Eos instead of an insufficient production of IL-2. Sharma et al. have reported that $Foxp3^+Eos^-$ Treg cells have more tendencies to switch their phenotype under autoimmune and inflammatory conditions, and that these cells could be further characterized as $CD4^+CD25^+Foxp3^+Eos^-CD38^+$ cells[136]. Interestingly, we found that the proportion of $Foxp3^+Eos^-CD38^+$ T cells was increased when the disease was established in our model. These findings further support a notion that the Treg cells in our model are phenotypically shifted.

To test the hypothesis that IL-35 could prevent MLDSTZ induced T1D, we injected IL-35 i.p. into MLDSTZ treated mice. Strikingly, we found that IL-35 treated mice did not develop hyperglycemia, whilst the control mice became diabetic. Furthermore, MLDSTZ + IL-35 treated mice did not develop T1D even though the IL-35 treatment was discontinued. To examine the effect of IL-35 on MLDSTZ mice, we studied the thymic glands, PDLNs and spleens of MLDSTZ + IL-35 and MLDSTZ + PBS treated mice. The proportions of $Foxp3^+$ Treg cells were not increased in MLDSTZ + IL-35

treated mice. These results were in contrast to previous finding since it has been reported that IL-35 triggers the proliferation of Treg cells[78,79]. Therefore, we determined the proportion of $Tbet^+$, IFN-γ^+ or IL-$17a^+$ cells among $Foxp3^+$ Treg cells, and found that the proportion of cells expressing these markers were decreased in MLDSTZ + IL-35 treated mice. Thus, these results indicate that IL-35 administration did not increase the number of Treg cells, but that it maintained the suppressive phenotype of the Treg cells and thus counteracted the development of hyperglycemia. This notion was further supported when we determined the expression of Eos in Treg cells and found that it was increased in MLDSTZ + IL-35 treated mice compared to controls. Our data thus suggest that IL-35 maintains the Treg cell phenotype by inducing the expression of Eos. To further confirm this, the lymphocytes of diabetic NOD mice were isolated from thymic glands, PDLNs and spleens. These isolated cells were treated with either IL-35 or PBS. We found that the proportion of IL-$17a^+$ cells was decreased and the Eos expression increased in IL-35 treated cells. Furthermore, the proportion of $Foxp3^+Eos^-CD38^+$ T cells was decreased in MLDSTZ+IL-35 treated mice. The numbers of Th1, Th17 and Tc cells were also decreased in MLDSTZ + IL-35 treated mice. The concentrations of serum insulin and IL-10 were increased by the MLDSTZ + IL-35 treatment. The degree of insulitis was also significantly lower and we observed a higher insulin positive staining in the pancreata of MLDSTZ+ IL-35 mice compared to MLDSTZ + PBS mice. In summary, these results indicate that IL-35 treatment maintains the Treg cells phenotype and keep the numbers of Th1, Th17 and Tc cells down in order to prevent the development of MLDSTZ induced T1D.

Next, we tested if IL-35 treatment could also reverse already established murine T1D in MLDSTZ mice. We let the mice become hyperglycemic and after two consecutive days of hyperglycemia, they were injected with IL-35 for 8 consecutive days. Three out of four mice remained normoglycemic even after discontinuing the treatment.

One can always argue that the response of Treg cells is strain biased and that this response is specific to MLDSTZ treated CD-1 mice. Therefore, we determined the proportion of both $Foxp3^+$ Treg cells and IFN-γ^+ cells among $Foxp3^+$ cells in female NOD mice and compared them with aged match CD-1 mice. The proportions of both cell populations were increased in female NOD mice, indicating that Treg cells are also increased in the NOD mouse model and that the Treg cells are phenotypically shifted.

We also treated new onset NOD mice with either PBS or IL-35 for 8 days, and followed them without treatment for the next 30 days. All NOD mice treated with IL-35 became normoglycemic, whilst the PBS treated mice

remained hyperglycemic. After discontinuing the treatment, 3 out of 6 mice reverted to diabetes. However, three of these mice remained normoglycemic until day 40 after the first injection. In summary, our results reveal that IL-35 can reverse established murine T1D and that perhaps this cytokine should be further studied for treating human T1D patients. This notion was further supported when we found decreased circulating levels of IL-35 in both T1D patients with a recent onset and long standing disease, compared to healthy controls.

Paper III

In this study we investigated why the majority of T1D patients with a long standing disease still have remaining residual C-peptide production. To answer this question we determined the circulating levels of pro- and anti-inflammatory cytokines in age, sex and BMI matched T1D patients with and without measurable C-peptide levels. The circulating levels of IL-35 were higher in C-peptide positive patients than in C-peptide negative patients. The higher levels of IL-35 in C-peptide positive patients suggest that these patients might have a higher tolerogenic response due to higher levels of IL-35, which might contribute to the prevention of the β-cell destruction. It has been reported earlier that ectopic expression of IL-35 in β-cells of NOD mice prevented the development of T1D in NOD mice [80]. Moreover, we have recently shown that systemic administration of IL-35 in diabetic MLDSTZ treated and NOD mice reversed established hyperglycemia [141]. Subsequently, we analyzed the proportions of IL-35$^+$ cells among Treg cells, Breg cells and tolerogenic APCs. The proportion of IL-35$^+$ cells among Treg cells was higher in C-peptide positive patients compared to C-peptide negative patients, illustrating that Treg cells of C-peptide positive patients produce more IL-35 compared to C-peptide negative patients. Next, the proportion of IL-17a$^+$ cells among Treg cells was determined, since it has been proposed that IL-35 maintains the phenotype of Treg cells [141]. The proportion of IL-17a$^+$ cells among Treg cells was higher in C-peptide negative patients, revealing that Treg cells of C-peptide negative patients lose their suppressive capacity due to a phenotypic shift. It seems worthwhile that IL-35 should be further investigated in patients with T1D.

Paper IV

In paper IV we elucidated the cellular immunology in patients with LADA, T1D and T2D. The proportion of APCs was lower in patients with LADA and T2D compared to T1D patients. The proportion of NK cells was higher in LADA and T1D patients than in T2D patients and healthy controls. It has earlier been shown that the depletion of NK cell in mice prevented them from the development of hyperglycemia [142,143]. The higher proportion of NK cells in patients with LADA suggests that the role of NK cells may be similar in LADA as in T1D. The proportion of Treg cells was not altered among the groups. However, the proportion of IL-35^{+} cells among Treg cells was lower in patients with LADA, T1D and T2D compared to health controls. A lower proportion of IL-35^{+} Treg cells in LADA, T1D and T2D patients implies that the diabetic patients have decreased numbers of IL-35^{+} Treg cells, which might contribute to the progression of disease. The proportion of Breg cells was higher in LADA patients compared to T1D, T2D and healthy controls. These data were in agreement with previous findings [65]. The proportion of IL-35^{+} cells among Breg cells was higher in LADA patients than in T1D patients. Furthermore, the proportion of IL-35^{+} cells were the lowest among APCs from T1D patients. It might be that the higher proportions of IL-35^{+} Breg cells and IL-35 $^{+}$ tolerogenic APCs leads to that LADA patients develop the autoimmune reaction later in life compared to T1D patients.

Paper V

To elucidate the role of Treg cells in patients with T1D with nephropathy, we determined the proportions of Treg cells in T1D patients with and without DN. These groups were age, sex and BMI matched with healthy controls. The proportions of Treg cells were lower in both T1D patients with and without DN compared to healthy controls. In addition, the proportions of IL-35$^+$ cells among Treg cells were lower in both T1D patients with and without DN compared to healthy controls. The proportions of IL-17a$^+$ cells among Treg cells were higher in both T1D patients with and without DN compared to healthy controls. Thus, these data indicate that the function of Treg cells is impaired in DN, like it is in T1D. To further investigate this, we examined the numbers of Foxp3$^+$ cells and the degree of inflammation in kidney tissues of MLDSTZ and vehicle treated mice on days 3, 7, 10 and 21 after the first injection of STZ. The mononuclear cell infiltration was significantly increased from day 7 and the number of Foxp3$^+$ cells was increased from day 10 in the kidneys of MLDSTZ treated mice compared to vehicle treated mice. An increase in the mononuclear cell infiltration in the kidneys of MLDSTZ treated mice on day 7 suggests that this response is due to an immune response, since the mice were normoglycemic at this time point.
In NOD mice, we found that the number of Foxp3$^+$ cells tended to increase in the kidney tubules at 10 weeks of age ($p = 0.065$), and that they were significantly increased at 16 weeks ($p = 0.012$), but not in 24 weeks old female mice when compared to 5 weeks old female mice. The mononuclear cell infiltration showed a similar pattern of increase i.e. these cells were increased at 10 weeks ($p = 0.065$), 16 weeks ($p = 0.012$) and 24 weeks ($p = 0.009$) mice compared to 5 weeks old NOD female mice. These results indicate that Foxp3$^+$ cells are increased in kidney tissues under T1D conditions, but that they did not prevent the mononuclear cell infiltration. Interestingly, when both MLDSTZ induced and NOD diabetic mice were treated with IL-35, we did not find any infiltrating mononuclear cells in the kidney tissues of IL-35 treated mice.

Conclusions

Paper I

- DCs and pDCs are increased at the early stage of T1D development in MLDSTZ treated mice.
- The response of DCs and pDCs may precede the response of neutrophils in the PDLNs of MLDSTZ treated mice.
- The B lymphocytes are altered during the early development of T1D in MLDSTZ treated mice.
- Both $CD4^{+}Helios^{+}$ and $CD8^{+}Helios^{+}$ cells are increased at an early stage of T1D development in MLDSTZ treated mice.

Paper II

- Both tTreg and pTreg cells are increased in MLDSTZ treated and NOD mice.
- Both tTreg and pTreg cells switch their phenotype in MLDSTZ treated and NOD mice.
- The Treg cells of MLDSTZ treated mice produced a decreased amount of the anti-inflammatory cytokines IL-10, IL-35 and TGF-β.
- The expression of Eos is impaired in diabetic MLDSTZ treated mice.
- IL-35 administration prevents the development of MLDSTZ induced T1D.
- IL-35 administration reverses already established T1D in both the MLDSTZ and NOD mouse models.
- IL-35 maintains the phenotype of Treg cells, seemingly by increasing the expression of Eos.
- The circulating levels of IL-35 are lower in T1D patients with both new onset and long standing disease compared to healthy subjects.

Paper III

- The circulating levels of IL-35 are increased in C-peptide positive T1D patients compared to C-peptide negative T1D patients.
- IL-35^{+} Treg cells are decreased in C-peptide negative T1D patients compared to both healthy controls and C-peptide positive T1D patients.
- IL-17a^{+} Treg cells are increased in C-peptide negative T1D patients compared to both healthy controls and C-peptide positive T1D patients.
- Th17 cells are increased in C-peptide negative T1D patients compared to both healthy controls and C-peptide positive T1D patients.
- IL-35^{+} Breg cells are decreased in C-peptide negative T1D patients compared to healthy controls.

Paper IV

- The cellular immune changes of APCs, IL-35^{+} tolerogenic APCs and IL-35^{+} Breg cells are similar in LADA patients to those observed in T2D patients.
- The response of NK cells in LADA patients is similar to that observed in T1D.
- The IL-35^{+} Treg cell response in LADA patients is similar to that observed in both T1D and T2D.

Paper V

- The numbers of Treg cells are lower in T1D patients with and without DN compared to healthy controls.
- The numbers of IL-35^{+} Treg cells are lower in T1D patients with and without DN compared to healthy controls.
- The numbers of IL-17^{+} Treg cells are higher in T1D patients with and without DN compared to healthy controls
- The numbers of Foxp3^{+} cells is increased in kidney tissue of both autoimmune and non-autoimmune induced hyperglycemia.
- The increase of Foxp3^{+} cell numbers cannot counteract the mononuclear cell infiltration in kidney tissue.
- The infiltrating mononuclear cells are increased before the numbers of Foxp3^{+} cells are increased in kidney tissues of autoimmune induced hyperglycemia.

Potential pitfalls of the study

In the present study, our hypothesis is generated based on findings in animal models, and of course there are limitations in translating such findings to human pathology. In our clinical investigations we are collecting immune cells from peripheral blood, and the extent to which this reflects immune cell reactions in the pancreas and the pancreatic islet compartment is unclear. By the same reasoning, it is very difficult to evaluate whether or not the time point when human samples are obtained is relevant to elucidate an on-going immune process of relevance for pathogenesis of type 1 diabetes.

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