Immunological strategies for counteracting type 1 diabetes focusing on IL-35 producing regulatory immune cells

ZHENGKANG LUO
Abstract

Type 1 diabetes (T1D) is an autoimmune disease where pancreatic β-cells are attacked by immune cells. Regulatory T (Treg) cells play critical roles in suppressing immune responses and their involvement have been intensively studied in T1D. Low dose IL-2 has been proposed to selectively boost Treg cells in T1D, with only limited success. We thus further decreased the IL-2 dosage and treated multiple low dose streptozotocin (MLDSTZ) mice with an ultra-low dose IL-2, but it did not protect STZ mice from hyperglycemia. Similarly, low dose IL-2 only partially prevented diabetes. Treg cells’ phenotype was not protected by either dose. These data suggest that alternative IL-2 therapies might be considered.

Regulatory B (Breg) cells suppress pro-inflammatory immune responses by producing anti-inflammatory cytokines IL-10 and IL-35. Decreased IL-35+ and increased IFN-γ+Breg cell proportions were found in T1D patients, and in diabetic mice. IL-35 treatment prevented increased IFN-γ+Breg cell proportions in STZ mice. These data illustrate Breg cells’ involvement in T1D, and IL-35 treatment prevents hyperglycemia by maintaining Breg cells’ phenotype.

Treg cells’ involvement in diabetic nephropathy (DN) has not been studied. Lower plasma IL-35 was found in DN patients than in T1D patients without DN and healthy controls, and was strongly correlated with kidney function. Decreased IL-35+ and increased IL-17+ Treg cells were found in DN patients. Moreover, Foxp3+ cell infiltration was found in the kidneys of diabetic mice, but it failed to counteract mononuclear cell infiltration. IL-35 treatment prevented DN and Treg cells’ phenotypic shift in STZ mice by maintaining the transcription factor Eos. These results demonstrate that IL-35 may be used to prevent DN.

Given the instability of IL-35, we explored the effect of IL-6 signaling blockade. Anti-IL-6R completely protected STZ mice from diabetes. Proteomics indicated enhanced metabolism and down-regulated pro-inflammatory pathways. It maintained Treg cells’ phenotype by increasing IL-35 and decreasing IFN-γ production. It also reduced the number of macrophages and conventional dendritic cells type 2 and their CD80 expression. STZ mice remained normoglycemic despite the discontinuation of anti-IL-6R treatment.

Therefore, our results illustrate the outcomes of several potential T1D immunotherapies and highlight the involvement of IL-35 producing immune cells in controlling the disease.

Keywords: Type 1 diabetes, Regulatory T cell, Regulatory B cell, Interleukin-35

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The sun has risen,
what’s the use of the lighthouse?
Long may the sun shine.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Publications not included in this thesis


*Equal contribution
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Abbreviations

ACR  Albumin/creatinine ratio
Breg cell  Regulatory B cell
cDC1  Conventional dendritic cell type 1
cDC2  Conventional dendritic cell type 2
DN  Diabetic nephropathy
eGFR  Estimated glomerular filtration rate
Foxp3  Forkhead box P3
GFR  Glomerular filtration rate
GLUT  Glucose transporter
IFN-γ  Interferon gamma
IL-2  Interleukin-2
IL-6  Interleukin-6
IL-6R  Interleukin-6 receptor
IL-10  Interleukin-10
IL-17  Interleukin-17
IL-35  Interleukin-35
iTreg cell  In vitro induced Treg cell
MLDSTZ  Multiple low dose streptozotocin
NOD  Non-obese diabetic
Nrp1  Neuropilin 1
PBMC  Peripheral blood mononuclear cell
pDC  Plasmacytoid dendritic cell
PDLN  Pancreatic draining lymph node
pTreg cell  Peripherally induced Treg cell
RA  Rheumatoid arthritis
SLE  Systemic lupus erythematosus
STZ  Streptozotocin
T1D  Type 1 diabetes
T2D  Type 2 diabetes
TGF-β  Transforming growth factor beta
Treg cell  Regulatory T cell
tTreg cell  Thymic derived Treg cell
Introduction

Diabetes mellitus

Glucose control is heavily dependent on insulin, which is a hormone produced by the β-cells in the pancreatic islets. Hyperglycemia occurs when insulin production is insufficient or the body cannot make good use of insulin, ultimately leading to diabetes mellitus. Today, diabetes has become a major health concern worldwide. It is estimated that 463 million people have diabetes worldwide in 2019\(^1\). The economic burden caused by diabetes is tremendous, as lifetime care and treatment are needed. Globally, the estimated diabetes-related health expenditure is 760 billion USD in 2019\(^2\).

Traditional symptoms of diabetes are polyuria, polydipsia, polyphagia and weight loss\(^3\). However, the occurrence of these symptoms may vary between individuals, thus laboratory tests are needed for diagnosis. The diagnostic criteria of diabetes include fasting plasma glucose \(\geq 7.0\) mmol/L, random plasma glucose \(\geq 11.1\) mmol/L, hemoglobin A1c \(\geq 48\) mmol/mol and 2 hour plasma glucose \(\geq 11.1\) mmol/L during oral glucose tolerance test\(^4\). Diabetes can be conventionally classified into type 1 diabetes (T1D), where β-cells are destroyed by autoimmune mechanisms leading to absolute insulin deficiency, and type 2 diabetes (T2D) where insulin production from β-cells is lost progressively due to insulin resistance\(^4\). Other types of diabetes are gestational diabetes mellitus, latent autoimmune diabetes in adults (LADA) and maturity-onset diabetes of the young (MODY)\(^4,5\). Patients with long term diabetes have high risk of developing diabetic complications, including nephropathy, retinopathy, neuropathy and cardiovascular diseases\(^6\).

It is estimated that type 1 diabetes accounts for 5-10% of total cases of diabetes\(^3\). In T1D, immune cells attack the β-cells by mistake, which leads to the destruction of β-cells and the insufficient production of insulin. The etiologies of T1D are not clear, but several factors have been related to its pathogenesis. Genetics, viral/bacterial infections and environmental factors have been shown to trigger the damage of β-cells\(^7\). It is hypothesized that an unknown event or a known event such as viruses causes β-cell damage and the release of islet antigens, which are presented to T cells by antigen presenting cells in the pancreatic draining lymph nodes\(^8\). Mononuclear cells are then recruited and infiltrate the islets, which is called insulitis. Effector T cells start the attack
to β-cells, leading to more β-cell destruction and the lack of insulin production. If no intervention is taken and this process continues, hyperglycemia eventually occurs.

Diabetic nephropathy

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease. Microalbuminuria is the earliest sign of DN manifestation, and approximately half of the patients with microalbuminuria will develop macroalbuminuria. Other features of DN include reduced glomerular filtration rate (GFR) and increased plasma creatinine.

Hyperglycemia in diabetes is a major risk of DN. An early study suggested that strict glycemic control normalizes the GFR in T1D patients. Blood sugar level and diabetes duration was found to be strongly correlated with microalbuminuria in T1D patients. As reviewed by Anders et al. hyperglycemia can cause immediate and long term kidney damage through a variety of mechanisms. However, hyperglycemia alone may not be the only explanation for the development of DN, since more than 60% of T1D patients are protected from developing DN by unknown factors despite that glomerulosclerosis is found in nearly all patients. Evidences have illustrated that inflammation and immune cells participate in the progression of DN. Monocytes, macrophages and T lymphocytes were found to play a major role in the inflammation in patients with diabetic glomerulosclerosis, which is considered to be an autoimmune process.

Regulatory T cell

The idea of suppressor T cells was proposed in 1970, when a study demonstrated suppressor immune cells derived from thymus. Attempts were made to find specific markers to distinguish this suppressor T cell type. In 1995, Sakaguchi and colleagues first showed that the IL-2 receptor α chain CD25 is constitutively expressed on T suppressor cells in mice, which provided a pioneering observation for the identification of human suppressor T cells. However, CD25 is not exclusively expressed on suppressor T cells, as it is also expressed on T cells upon activation. The transcription factor forkhead box P3 (Foxp3) was discovered in 2001, and its mutation was found to cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in human. In 2003, several studies indicated that Foxp3 is the master regulator of CD4⁺CD25⁺ suppressor T cells. Since then CD4⁺CD25⁺Foxp3⁺ cells are called regulatory T (Treg) cells. Treg cells play a central role in immune tolerance by exerting a suppressive ability via a
variety of mechanisms including cytolysis through the secretion of granzymes to induce apoptosis, cytokine consumption and inhibition through the expression of cytotoxic T-lymphocyte antigen (TGF-β)17–20. Additionally, they can secrete inhibitory cytokines such as transforming growth factor beta (TGF-β), interleukin-10 (IL-10) and interleukin-35 (IL-35)21.

Treg cells can naturally be derived either from the thymus, which are designated thymic derived Treg (tTreg) cells. Alternatively, another population of Treg cells can be generated from Foxp3+ conventional T cells in the periphery22, in which case they are termed peripherally induced Treg (pTreg) cells. Moreover, Treg cells can also be generated in vitro from CD4+CD25− cells by TGF-β23, thus they are called in vitro induced Treg (iTreg) cells. Thornton et al. suggested Helios, a member of Ikaros transcription factor family, as a marker for tTreg cells24. Following studies suggested that neuropilin 1 (Nrp1) is a specific marker for tTreg cells25,26. However, results from our group showed that Helios is a better marker for tTreg cells than Nrp127. Furthermore, Nrp1+ Treg cell percentage is dependent on TGF-β derived from Treg cells28. Thus, we chose Helios as a marker for tTreg cells in our study, and considered tTreg cells as CD4+CD25+Foxp3+Helios+ cells and pTreg cells as CD4+CD25+Foxp3+Helios− cells.

Despite their suppressive ability, Treg cells are not always functional. The defective functions of Treg cells have been found in many immune related diseases, such as amyotrophic lateral sclerosis and dilated cardiomyopathy29,30. In autoimmune diseases, it seems very common that the Treg cells are not functional, which has been reported in multiple sclerosis, ankylosing spondylitis, celiac disease and type 1 diabetes31–33. Dysfunctional Treg cells produce less anti-inflammatory cytokines (IL-10 and IL-35) and start to produce pro-inflammatory cytokines such as interferon gamma (IFN-γ) and interleukin-17 (IL-17)34.

Regulatory B cell

The idea of B cell’s suppressive function was first claimed in 1974, when a study demonstrated that B cells or their product(s) suppress the expression of delayed hypersensitivity reactions35. Another study in 1996 showed that B cell-deficient mice failed to recover from experimental autoimmune encephalomyelitis36. Following studies characterized regulatory B (Breg) cells as IL-10 producing B cells, which could also produce IL-35 and TGF-β37–39. From 2002, this immunosuppressive cell type was well recognized for the discovery in mouse models of its ability to suppress inflammation in colitis, experimental autoimmune encephalomyelitis and collagen-induced arthritis40–42. Breg cells were also shown to maintain the tolerance to islet autoantigens in
NOD mice\textsuperscript{46}. Despite all efforts, little is known about the role of Breg cells in patients with autoimmune diseases. Breg cells isolated from peripheral blood of systemic lupus erythematosus patients produced less IL-10 than those from healthy individuals and lost the suppressive capacity\textsuperscript{47}. Similar to Treg cells, Breg cells can suppress the differentiation of pro-inflammatory immune cells through the production of anti-inflammatory cytokines. Unlike Treg cells, no specific Breg cell transcription factor has been identified yet. Multiple IL-10-producing Breg cell subsets that have similar phenotypes have been described, but the up- or downregulation of the surface markers used to identify Breg cells lead to a difficulty in defining different Breg cell subsets in different experimental settings\textsuperscript{41}. Among all the Breg cell subsets, the CD19$^+$CD1d$^+$CD5$^+$ subset is most studied in mice. This subset of Breg cells was first identified by Yanaba and colleagues in 2008\textsuperscript{48}. Though CD19$^+$CD1d$^+$CD5$^+$ Breg cells are predominantly found in the spleen, they can also be found in lymph nodes and thymus\textsuperscript{48,49}. Although Breg cells are defined as IL-10-producing B cells, their ability to produce IL-35 has been shown to be critical in autoimmune diseases\textsuperscript{40,42}. In an experimental autoimmune uveitis mouse model, IL-35 was reported to induce IL-10- and IL-35-producing Breg cells\textsuperscript{40}. Furthermore, Wang et al reported that IL-35 reversed established autoimmune uveitis and prevented its development through IL-35 signaling and IL-10, indicating the significant role of IL-35 in Breg function.

**IL-2**

IL-2 was discovered and cloned more than 40 years ago, and it was initially considered as a T cell growth factor for its capacity of stimulating T cell proliferation in vitro\textsuperscript{50,51}. It is predominately produced by activated CD4$^+$ T cells, and by CD8$^+$ T cells to a lesser extent\textsuperscript{52}. IL-2 is important for both CD4$^+$ T and CD8$^+$ T cells, controlling their proliferation and differentiation and exerting other variable roles in T cell immunity, as reviewed by Boyman and Sprent\textsuperscript{53}. However, the lack of IL-2 was found to cause autoimmunity, rather than the expected immunodeficiency. For example, a study on IL-2 deficient mice showed that all the IL-2$^{-/}$ mice that died after 9 weeks of age developed an inflammatory bowel disease similar to ulcerative colitis in human\textsuperscript{54}. Uncontrolled activation and proliferation of CD4$^+$ T cells and autoimmunity were observed in IL-2 deficient BALB/c mice\textsuperscript{55}. It was later found out that such autoimmunity was due to the deficiency or dysfunction of Treg cells\textsuperscript{56}. IL-2 signaling is required for CD4$^+$CCR7$^+$Helios$^+$ cells to avoid deletion in the thymus and for the cells to further differentiate into Treg cells\textsuperscript{57}. Although Treg cell survival is independent of IL-2, it is indispensable for the suppressive capacity of Treg cells\textsuperscript{58}. 
The IL-2 receptor (IL-2R) is composed of three chains: an α chain (IL-2Rα, CD25), a β chain (IL-2Rβ, CD122) and a γ chain (also known as the common γ chain, γc, CD132). When all the three chains are co-expressed on a cell, IL-2 is bound with high affinity\(^5^9\). In the absence of CD25, IL-2Rβ and γc form a complex (IL-2Rβγ), which binds IL-2 with intermediate affinity\(^5^9\). If CD25 is expressed alone, the cell has a low affinity to IL-2 binding. Due to the difference in affinity to IL-2 binding among the IL-2R complexes, different doses of IL-2 can be given to trigger different IL-2R signaling. When a high dose of IL-2 is given, all IL-2 receptors are activated regardless of the affinity. However, the low affinity IL-2R cannot be activated when there is only low dose of IL-2.

IL-2 was originally used for cancer therapy. High doses of recombinant human IL-2 was injected to patients with metastatic cancer, which, to the best of our knowledge, was the first report showing that high dose IL-2 treatment could induce the regression of cancer\(^6^0\). Besides Treg cells, CD4\(^+\) T cells, CD8\(^+\) T cells, B cells, eosinophils and innate lymphoid cells also express intermediate affinity IL-2R, which, in the context of autoimmune therapy where the Treg cell population is expected to be expanded, will lead to the unspecific IL-2 binding. CD25 is constitutively expressed on Treg cells, and thus the high affinity IL-2R is expressed on Treg cells. Therefore, low dose IL-2 treatment has been proposed to selectively boost Treg cells and is used to treat diseases in clinical trials such as graft-versus-host disease, HCV-induced vasculitis and systemic lupus erythematosus (SLE)\(^6^1-6^3\). In a T1D mouse model, low dose IL-2 administration prevented diabetes in NOD mice and partially induced diabetes remission\(^6^4,6^5\). Therefore, several clinical trials have been conducted in T1D\(^6^6-6^9\). As expected, low dose IL-2 induced the increase in Treg cell proportion in all these trials. However, significant improvement of metabolic control was only observed in one trial\(^6^7\), and improved maintenance of induced C-peptide was observed in another trial\(^6^9\).

**IL-35**

As a new member of IL-12 family, IL-35 is a heterodimeric cytokine composed of two subunits: the Epstein-Barr virus-induced gene 3 (Ebi3) and the p35 subunit of IL-12 (IL-12p35)\(^7^0-7^2\). Unlike the other members in this family (IL-12, IL-23 and IL-27), IL-35 is a potent inhibitory cytokine, which is mainly produced by Treg cells\(^7^3\). It has been shown that Breg cells and tolerogenic dendritic cells can also produce IL-35\(^4^0,4^2,7^4\). Given the suppressive ability of IL-35, its roles in a variety of diseases have been investigated. Elevated IL-35 levels have been found in cancers, like colorectal cancer, hepatocellular carcinoma and acute myeloid leukemia\(^7^5-7^7\). Growing evidence suggests that the lack of IL-35 contributes to the loss of immunological self-
tolerance in several autoimmune diseases, such as primary immune thrombocytopenia, rheumatoid arthritis and T1D\textsuperscript{37,78,79}. Our previous results demonstrated decreased plasma IL-35 levels in patients with diabetes\textsuperscript{80}, and indicated a protective effect of IL-35 on β-cells\textsuperscript{81}. In different animal models, IL-35 treatment offered promising data in acute graft-versus-host disease, experimental autoimmune uveitis and T1D\textsuperscript{37,40,82-84}. The IL-35 receptor is a heterodimer composed of gp130 and IL-12Rβ2 on Treg cells, with Ebi3 binding to gp130 and IL-12p35 to IL-12Rβ2. The homodimer of gp130 or IL-12Rβ2 is sufficient for IL-35-mediated suppression, but the heterodimer shows the strongest IL-35 signaling\textsuperscript{85}. While on murine Breg cells, IL-35 signals through the heterodimer of IL-27Rα and IL-12Rβ2\textsuperscript{40}. The IL-35 receptors on tolerogenic dendritic cells have not been identified yet.

**IL-6**

IL-6 was initially identified as a B cell stimulator for its ability to differentiate B cells into antibody producing cells\textsuperscript{86}. It is produced by a wide range of immune and non-immune cells upon stimulation in response to emergent events\textsuperscript{87,88}. Later studies revealed that IL-6 is a pleiotropic cytokine with multiple physiological and pathological roles. Its overproduction was found to contribute to the pathogenesis of several inflammatory and autoimmune diseases\textsuperscript{89}. The elevation of IL-6 level has been found in autoimmune diseases such as rheumatoid arthritis (RA)\textsuperscript{90}, SLE\textsuperscript{91}, systemic sclerosis\textsuperscript{92}, Crohn's disease\textsuperscript{93}, and T1D\textsuperscript{94}. IL-6’s role extends beyond immunity as it is also an important regulator of glucose and lipid metabolism\textsuperscript{95,96}. IL-6 released from adipose tissue was found to increase expression of liver SOCS3 which induces hepatic insulin resistance\textsuperscript{97}, while another study demonstrated that exercise induced IL-6 increased GLP-1 which improves insulin resistance and induces β-cell expansion\textsuperscript{98}. In terms of lipid metabolism, IL-6 has been shown to reduce the levels of total cholesterol and triglyceride in the circulation\textsuperscript{99,100}.

IL-6 binds to a membrane-bond or soluble IL-6R to form a complex\textsuperscript{101}, and this complex binds to the signal transducer gp130\textsuperscript{102,103}. Due to the utilization of gp130 in its receptor, IL-35 can also be characterized as an IL-6 family cytokine\textsuperscript{104}. Notably, gp130 only interacts with the IL-6/IL-6R complex with high affinity but not with the individual proteins\textsuperscript{102,103}. This enables a possible therapeutic strategy of blocking the formation of IL-6/IL-6R complex thus the blockade of IL-6 signaling to treat IL-6 mediated diseases. Early studies have shown that anti-IL-6R suppressed the diseases in mouse models of RA\textsuperscript{105}, SLE\textsuperscript{106} and Castleman’s disease\textsuperscript{99}. A human anti-IL-6R tocilizumab was later developed by Kishimoto and Chugai Pharmaceutical Co. in the 2000s, and it was initially approved for the treatment of RA. Much progress has been made
in treating RA patients with tocilizumab, and research on using it in other hu-
man diseases is still going on\textsuperscript{107}.
Aims

The aim of this work is to elucidate the role of IL-35 and IL-35 producing regulatory immune cells in T1D. The specific aims of each paper are:

1. To find an optimal IL-2 dose for low dose IL-2 treatment in experimental T1D and evaluate its efficacy.
2. To investigate the role of IL-35 producing Breg cells in T1D and potential mechanisms of IL-35 treatment.
3. To investigate the role of IL-35 producing Treg cells in diabetic nephropathy and the effect of IL-35 treatment.
4. To explore the effect of anti-IL-6R on metabolism and immune cells in experimental T1D.
Methods and materials

Animals
All animal experiments were approved by the animal ethical board of the Uppsala Region. All mice were housed and bred in pathogen–free conditions in the animal facility in the Biomedical Centre, Uppsala University, Uppsala.

MLDSTZ mouse model
Streptozotocin (STZ) was first identified as an antibiotic\textsuperscript{108}. It has a similar structure to glucose, so it can be transported via GLUT2\textsuperscript{109}. Due to its toxicity to pancreatic $\beta$-cells, this drug was used to treat islet-cell tumors and to induce diabetes in animal models\textsuperscript{110,111}. It was later found that STZ could cause DNA damage of $\beta$-cells in vitro and in vivo\textsuperscript{112,113}. The dosage of STZ varies depending on the specific purposes of different studies. A single high dose of STZ has been reported to cause an almost complete destruction of the $\beta$-cells\textsuperscript{114}, with only 10% of the mice showing mononuclear cell infiltration in the islet of Langerhans\textsuperscript{115}. On the other hand, multiple low doses of STZ are able to induce insulitis and considered as an inducer of autoimmune diabetes\textsuperscript{116,117}. Five consecutive low doses of STZ can trigger pancreatic insulitis\textsuperscript{37,118}. However, this response is both strain and sex biased in mice\textsuperscript{116}. Male CD-1 mice are more susceptible to STZ injections than female mice, and the CD-1 mouse strain has the highest response to STZ injections than other mouse strains\textsuperscript{116,119}.

STZ induced hyperglycemia is widely used to study experimental diabetes and DN. However, due to the renal toxicity of STZ\textsuperscript{120}, concerns exist whether STZ models are suitable for the study of DN. By transplanting $\beta$-cells to STZ treated rats, researchers demonstrated that parameters including GFR, urinary albumin excretion are caused by diabetes condition, while urinary protein leakage other than albumin is partially due to the nephrotoxicity of STZ\textsuperscript{121}. Therefore, the MLDSTZ model was used in our studies.

In our present studies, male CD-1 mice were obtained from Charles River (Hannover, Germany). They received intraperitoneal injections of STZ
(Sigma-Aldrich, St Louise, MO, USA; 40 mg/kg body weight) dissolved in 200 µl saline for 5 consecutive days.

NOD mouse model
Unlike MLDSTZ mice which develop hyperglycemia gradually after induction with STZ, NOD mice develop diabetes spontaneously. Sex difference exists in the NOD mouse model. The occurrence of diabetes happens much later in male NOD mice than in females, and the cumulative incidence of diabetes onset up to 30 weeks is 80% in females but less than 20% in males. In female NOD mice, insulitis is observed at high incidence after 5 weeks of age, and the onset of diabetes is observed at approximately 10 weeks of age.

The NOD mice used in our studies were originally obtained from the Clea Company (Aobadai, Japan). The mice have thereafter been inbred in the animal facility at the Biomedical Centre, Uppsala, Sweden.

Cytokine and antibody treatment
Male CD-1 mice or female NOD mice received intraperitoneal injections of IL-2 (Mouse IL-2 Carrier-Free Recombinant Protein, #34-8021-82, eBioscience, San Diego, CA, USA; or Recombinant Mouse IL-2 Protein, #402-ML/CF, R&D Systems, Minneapolis, MN, USA), IL-35 (recombinant mouse IL-35, #CHI-MF-11135-C025, Chimerigen, Liestal, Switzerland) or anti-IL-6R (#BE0047, Bio X Cell, Lebanon, NH, USA) dissolved in 200 µl PBS. Other mice received 200 µl PBS injection as comparison. Details on each treatment are provided in paper I-IV.

Blood glucose measurements
Blood was obtained from the tail vein of the mice and the blood glucose levels were measured using a blood glucose meter (FreeStyle Freedom Lite, Abbott). The mice with blood glucose levels above 11.1 mM were considered hyperglycemic.

Tissue preparation
Thymi, pancreatic draining lymph nodes (PDLNs), spleens, pancreata and kidneys were removed from the mice after they were killed by cervical
dislocation. Spleens, thymi and kidneys were then placed in glass jars or 6-well plates filled with 5 ml Hanks’ balanced salt solution (HBSS, Statens veterinärmedicinska anstalt) while PDLNs were placed in Eppendorf tubes filled with 1 ml RPMI 1640 (Sigma Aldrich). Pancreata and kidneys were transferred to 10% formalin for morphological analysis.

Cell isolation from thymi, PDLNs, spleens and kidneys

Cell isolation from thymi, spleens and PDLNs were prepared as described\textsuperscript{124}. The thymic glands and spleens were squeezed with a pair of tweezers or glass slides. The cell suspension was then transferred to a 15 ml Falcon tube and centrifuged to form a pellet. The cell pellet was re-suspended in 5 ml 0.2M NH\textsubscript{4}Cl and incubated at room temperature for 10 min and mixed manually every 2 minutes to lyse red blood cells. Then the tubes were centrifuged and washed with 15 ml HBSS twice before being re-suspended in 5 ml HBSS. PDLNs were grinded with a pair of tweezers on a sterile mesh rinsed with RPMI-1640 or squeezed with a pair of glass slides. The cell suspension was centrifuged and washed with 5 ml RPMI-1640 twice followed by being re-suspended with 2 ml RPMI-1640.

The kidneys were transferred to a petri dish with 5 ml HBSS and cut into small pieces with a pair of scissors. They were then placed on a 40 µm cell strainer put on a 50 ml Falcon tube, crushed by a syringe plunger and rinsed with 1 ml HBSS five times. The suspension was then centrifuged to form a pellet. The cell pellet was re-suspended in 10 ml 0.2M NH\textsubscript{4}Cl and incubated at room temperature for 10 min and mixed manually every 2 minutes to lyse red blood cells. The tubes were then centrifuged and washed with 20 ml HBSS before being re-suspended in 10 ml HBSS.

Flow cytometry

Cell surface and intracellular staining were performed as described\textsuperscript{124}. Details about antibodies used are provided in corresponding papers. All the samples were analyzed using a BD LSR Fortessa at the BioVis Platform (Uppsala University, Uppsala, Sweden). All data from flow cytometry were analyzed using the Flowlogic software (Inivai Technologies, Australia) or FlowJo (Ashland, OR, USA).
In vitro stimulation

Splenocytes were prepared as aforementioned. CD19 cells were enriched using CD19 MicroBeads (#130-121-301, Miltenyi Biotec, Germany). Approximately 2 million CD19 cells were then cultured in 6-well plates containing RPMI-1640 supplemented with fetal bovine serum and penicillin. Cells were stimulated with CD40L (0.1 mg/ml, Thermo Fisher) and LPS (1 µl/ml, Sigma-Aldrich) for 72h. Culture supernatants were saved for ELISA experiments. Next, PMA (50ng/ml, Sigma-Aldrich) and ionomycin (1 µM, Sigma-Aldrich) were added to the culture for 5h. Cells were analyzed using flow cytometry.

Cell sorting

CD19+CD1d+CD5+ Breg cells were stained with surface antibodies (paper II) and sorted using BD FACSariaIII at the BioVis Platform, Uppsala University.

Mass spectrometry

Details about mass spectrometry are provided in paper IV. In short, digested peptides from mouse sera were analyzed using an UltiMate 3000 UHPLC system interfaced to a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific). The DIA-NN software was operated in library-free mode for direct analysis and quantification of data-independent acquisition datasets\textsuperscript{125}. Downstream differential expression analysis was processed by the DEqMS R package\textsuperscript{126}. Over-representation analysis and gene set enrichment analysis of the relevant proteins/genes were conducted using the clusterProfiler R package\textsuperscript{127}, along with its associated packages.

ELISA

The concentrations of IFN-γ and IL-35 in the culture supernatants were measured using LEGEND MAX\textsuperscript{TM} Mouse IFN-γ ELISA Kit (#430807, BioLegend, San Diego, CA, USA) and ELISA Kit for Interleukin 35 (SEC008Mu, Cloud-Clone, Wuhan, China) following the manufacturer’s instructions.

Human IL-35 plasma concentrations were measured using the ELISA kit for IL-35 (Cloud-Clone) following the manufacturer’s instructions.
Quantitative RT-PCR

qRT-PCR was performed using SingleShot™ SYBR® Green One-Step Kit (Bio-Rad, #1725095) following the manufacturer’s instructions. All samples were performed using QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). All primers were obtained from IDT. Information of primers used is provided in paper II.

Morphological analysis

Pancreatic and kidney tissue were fixed in 10% formalin for 24 hours. Tissues were embedded in paraffin, and then sectioned at 5-7 µm thickness using a microtome (Thermo Scientific Microm HM355S Rotary Microtome, Walldorf, Germany) as described earlier37. Five to six sections were discarded between each section to cover the entire tissue area and to avoid including the same cells in consecutive sections.

The Foxp3 staining was performed as previously described37,128. The insulin staining was performed as previously described129. The slides were analyzed in a blinded manner under a light microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany; or Olympus BX53, Olympus Corporation, Tokyo, Japan). The degree of insulitis and number of Foxp3+ cells were graded as previously described37. The arbitrary scoring information is provided in the corresponding papers.

Albumin and creatinine determination in urine

Urine samples from mice were analyzed for albumin (Mouse Albumin ELISA Kit; Bethyl Laboratories, Montgomery, TX) and creatinine concentrations (Abbott Laboratories, Abbott Park, IL). Analysis was performed according to instructions from manufacturer. The concentration of albumin in urine was normalized to the concentration of creatinine in the same sample to avoid erroneous conclusions of albuminuria primarily based on changes in glomerular filtration rate (Albumin/Creatinine ratio, ACR).

Human whole blood and plasma samples

All human sample analysis was approved by the Uppsala County regional ethics board and was carried out in accordance to the principles of the Declaration of Helsinki. T1D patients and healthy individuals (HC) were recruited at Uppsala University Hospital. All participants received oral and written
information and signed a written consent form. Peripheral blood was collected. Freshly isolated peripheral blood mononuclear cells (PBMCs) were prepared using Histopaque-1077 (Sigma-Aldrich). PBMCs were then washed with RPMI-1640 twice before antibody staining. Detailed information on antibodies are provided in papers II-III.

Statistical analysis
The results are expressed as means ± SEM. The GraphPad Prism software version 7 and 9 was used for the statistical analysis. Pearson’s correlation was used for correlation analyses. Log-rank test followed by Bonferroni correction was used for survival curves. Unpaired t-test, one-way ANOVA followed by Tukey’s test, repeated measurement two-way ANOVA followed by Tukey’s test, repeated measurement two-way ANOVA followed by Šidák’s test were used for parametric comparisons. Mann-Whitney test and Kruskal-Wallis test followed by Dunn’s test were performed for nonparametric comparisons. A p-value below 0.05 was considered statistically significant. Detailed information on what tests were used for each experiment is provided in the figure legends of papers I-IV.
Results and discussion

Paper I

Low dose IL-2 has been proposed to selectively boost Treg cells due to their constitutive expression of IL-2Rα (CD25). However, only limited success has been seen in clinical trials, we therefore explored the possibility of further decreasing the IL-2 dosage for better a responsiveness of Treg cells.

STZ mice received ultra-low dose IL-2 (uIL-2, approximately 7-fold lower than low dose) treatment after STZ injections. We found that uIL-2 did not protect STZ mice from developing hyperglycemia, and no improvement of insulitis was seen. uIL-2 did not increase the proportions of Treg cells, nor did it maintain the phenotype of Treg cells. It only partially reduced IFN-γ production in T cells. Similarly, uIL-2 did not rescue Breg cells from decreased IL-35 and IL-10 production. IL-35 has been shown to protect STZ mice from hyperglycemia\(^{37}\). Surprisingly, uIL-2 also abrogated the protective effect of IL-35.

On the other hand, the low dose IL-2 (ldIL-2) partially prevented hyperglycemia as previously reported\(^{64}\), and it counteracted insulitis. No difference in the proportions or IL-35 production of Treg and Breg cells was found, but ldIL-2 tended to reduce CD80 expression in macrophages and cDC1s.

Although early work has shown the protective effect of low dose IL-2 in NOD mice\(^{64}\), several clinical trials showed that low dose IL-2 was able to increase Treg cell frequency but only a limited improvement on metabolic control was found\(^{66-69}\). Despite the idea of decreasing IL-2 dose looks promising, our results showed that uIL-2 could not prevent diabetes in STZ mice. Even though ldIL-2 protected half of the mice from diabetes as previously reported\(^{64}\), this result is far from effective when compared with the almost complete protection provided by IL-35. In summary, further decreasing IL-2 dosage may not be a successful T1D therapy, whilst other low dose IL-2 therapy strategies could be considered.
Paper II

To investigate the participation of Breg cells in T1D, we collected PBMCs of T1D patients and HCs. The proportions of Breg cells and IL-35$^+$ cells among Breg cells were lower in T1D patients than in HCs, but the proportions of IFN-$\gamma^+$ cells among Breg cells were significantly higher in T1D patients than in HCs.

We further studied Breg cells in NOD and MLDSTZ models. We found that the proportions of Breg cells were decreased in the spleens of 18-week-old NOD mice compared with 8-week-old mice. Lower proportions of IL-35$^+$ cells among Breg cells were found in the PDLNs and spleens of older NOD mice. A previous study reported IL-35 production from CD138$^+$ plasma cells$^{42}$, and we found decreased proportions of IL-35$^+$ cells among CD19$^+$CD138$^+$ cells in the PDLNs and spleens of older NOD mice.

Male CD-1 mice were injected with low dose STZ for five consecutive days, and they developed hyperglycemia gradually. An initial increase of Breg and IL-35 Breg cell response was found in MLDSTZ mice at the early stage of hyperglycemia development, but such increase turned into decrease in established experimental T1D on day 21. Such transient compensation of Breg IL-35 and its failure to prevent the development of hyperglycemia made us investigate the effect of IL-35 treatment on these mice, as we have shown that IL-35 treatment prevented the occurrence of experimental T1D by maintaining the phenotype of Treg cells$^{37}$. Here we found IL-35 treatment restored the proportions of Breg cells and their ability to produce IL-35. We further determined whether Breg cells were phenotypically shifted. Higher proportions of IFN-$\gamma^+$ cells among Breg cells were found in MLDSTZ mice, and IL-35 treatment prevented this phenotypic shift.

Thus, our results indicate that the Breg proportion and their ability to produce IL-35 are impaired in T1D. Our previous study showed that IL-35 treatment prevented the development of hyperglycemia and reversed established hyperglycemia by maintaining Treg cell phenotype and suppressing T cell response$^{37}$. Another study showed that IL-35 treatment suppressed autoimmune uveitis, expanded Breg cells and induced IL-35 production from Breg cells$^{40}$. Herein, we extend the suppressive function of IL-35 in T1D to IL-35 producing Breg cells, and IL-35 treatment effectively maintained the anti-inflammatory phenotype of Breg cells in the T1D mouse model.
Paper III

We recruited T1D patients with and without DN and HCs and collected their PBMCs and plasma samples. We found that both T1D groups had lower plasma IL-35 levels than HCs, while DN patients had the lowest IL-35 levels. We also found IL-35 plasma levels showed a strong negative correlation with creatinine and positive correlation with estimated glomerular filtration rate (eGFR). The proportions of Treg, tTreg and pTreg cells were decreased in the PBMCs of both T1D groups compared with HCs. Moreover, we found the phenotypic shift of Treg, tTreg and pTreg cells in both T1D groups based on the decreased IL-35+ and increased IL-17+ cell proportions among them. Interestingly, these Treg cells were more phenotypically shifted in T1D patients with DN than without DN.

We next used MLDSTZ and NOD models. More Foxp3+ cells were observed in the kidneys of both MLDSTZ and older NOD mice, but they failed to stop the infiltration of mononuclear cells (MNCs), suggesting these Foxp3+ cells were dysfunctional. Indeed, we found higher proportions of Treg cells in the kidneys of MLDSTZ mice on day 21, but decreased proportions of IL-35+ cells among Treg cells. Thus, we treated mice with IL-35. This treatment prevented the elevated ACR, maintained the phenotype of Treg cells in the kidney by maintaining the transcription factor Eos. IL-35 also reduced the infiltration of Foxp3+ cell in the kidneys of MLDSTZ mice and reduced MNC infiltration in the kidneys of both MLDSTZ and NOD mice.

Hyperglycemia is a known factor of DN, but it is still not clear why most of T1D patients are protected from developing DN. Our results illustrated that although T1D patients have lower systemic IL-35 levels than HCs, the remaining IL-35 seems to be enough to protect them from developing DN.

The protective role of Treg cells in DN was demonstrated in the db/db mouse model, where the intravenous transfer of Treg cells improved insulin sensitivity and prevented the development of DN. However in our study, Treg cells failed to prevent the development of DN because they were phenotypic shifted. Previous studies have demonstrated that the transcription factor Eos is important in maintaining the phenotype and suppressive function of Treg cells. Our current findings indicate that IL-35 treatment could prevent the development of DN by maintaining Eos in Treg cells in the kidney.

Researchers have suggested a more inflamed condition in T1D patients with DN than without. Our findings indicate that Treg cells in DN patients are even more phenotypically shifted on top of the already existing phenotypic shift of Treg cells in T1D, which could further explain why some T1D patients
develop autoimmune diabetic complications including DN while others do not.

Taken together, our study demonstrates that Treg cells are phenotypically shifted in DN, and IL-35 treatment can prevent the development of DN in two T1D mouse models.
Paper IV

The instability of IL-35 currently limits its application in treating human diseases\textsuperscript{136}. Because IL-6 and IL-35 both utilize gp130 in their receptors, it is plausible that blocking IL-6R would result in a competitive increase in IL-35 signaling. We therefore treated STZ mice with anti-IL-6R and investigated its effect on metabolic control and immune response.

We found that none of the mice treated with anti-IL-6R became diabetic. Pancreatic sections were made, and immunostaining indicated that the ability to produce insulin was preserved by anti-IL-6R with almost no insulitis being observed. Sera of the mice were saved and analyzed by mass spectrometry. Proteomics data indicated restored metabolism and a downregulation of several pro-inflammatory pathways.

The immune response was next investigated using flow cytometry, since serum proteins may hinder the detection of immunologically relevant proteins in the serum using mass spectrometry. Increased proportions of Treg cells were found in STZ mice as previously reported\textsuperscript{37}, but no such increase was found in anti-IL-6R treated mice. As expected, anti-IL-6R increased the expression of Ebi3 in Treg cells. It maintained the phenotype of Treg cells by increasing IL-35 production and decreasing IFN-\(\gamma\) expression. Similarly, IFN-\(\gamma\) production was reduced in CD4\(^+\) and CD8\(^+\) T cells.

Even though reduced IFN-\(\gamma\) in B cells was found in, anti-IL-6R only partially increased IL-35 production in Breg cells. Increased numbers of macrophages and conventional dendritic cells type 2 (cDC2s) were seen in STZ mice, and they were reduced by anti-IL-6R. Likewise, increased expression of CD80 was found in macrophages and cDC2s in STZ mice, and anti-IL-6R decreased CD80 expression in these cells. These data suggest that anti-IL-6R maintained the phenotype of Treg cells and limited the activation of co-stimulatory pathway on macrophages and cDC2s.

The long-term effect of anti-IL-6R was then investigated. Twenty-five days after the last anti-IL-6R treatment, STZ mice remained normoglycemic and no insulitis was seen. Indeed, the antagonistic effect of anti-IL-6R was diminishing as we could only find a decreasing trend of IL-6R expression on multiple immune cell types. These results, however, suggested that the blockade of IL-6R at an early stage of T1D development is enough to maintain normal blood glucose.
Conclusions

Paper I

- Ultra-low dose IL-2 does not protect against hyperglycemia in STZ mice.
- Ultra-low dose IL-2 does not maintain the function of Treg and Breg cells.
- Low dose IL-2 partially protects against hyperglycemia in STZ mice.
- Low dose IL-2 tends to reduce CD80 expression on antigen presenting cells.

Paper II

- Proportions of Breg cells and IL-35\(^+\) Breg cells are decreased in PBMCs of T1D patients, while IFN-\(\gamma\)^+ Breg cells are increased.
- In both NOD and MLDSTZ mice, IL-35\(^+\) Breg cells are increased at the early stage of disease development, but decreased when experimental T1D is established.
- The impairment of IL-35 receptors on Breg cells may contribute to the failure of IL-35 feedback loop of Breg cells.
- IL-35 treatment prevents the decrease of Breg and IL-35\(^+\) Breg cells.
- IFN-\(\gamma\)^+ Breg cells are increased at the early stage of T1D development in MLDSTZ mice, which can be prevented by IL-35 treatment.

Paper III

- DN patients have the lowest plasma IL-35 levels, and the IL-35 concentration is strongly correlated with kidney function.
- T1D patients have lower proportions of Treg cells in the PBMCs, and DN patients have the lowest proportions of IL-35\(^+\) Treg cells.
• The proportions of IL-17\(^+\) Treg cells are higher in T1D patients with or without DN than in HC.

• The numbers of infiltrating Foxp3\(^+\) cells are increased in the kidneys of T1D mouse models, but these cells cannot counteract the infiltration of MNCs in kidney tissues.

• Treg cell proportions are increased in kidneys of MLDSTZ mice, but IL-35\(^+\) Treg cell proportions are decreased.

• Treg cells are phenotypically shifted in the kidneys of MLDSTZ mice, and IL-35 treatment prevents the phenotypic shift by maintaining transcription factor Eos.

• IL-35 treatment prevents the infiltration of Foxp3\(^+\) cells and MNCs into the kidneys of MLDSTZ and NOD mice.

Paper IV

• Anti-IL-6R completely prevents STZ mice from developing hyperglycemia and almost no insulitis is observed.

• Proteomics data indicate metabolic benefits and down-regulated pro-inflammatory pathways by anti-IL-6R treatment.

• Anti-IL-6R elevates Ebi3 expression in Treg cells and maintains the phenotype of Treg and Breg cells.

• Anti-IL-6R reduces the expression of CD80 on macrophages and cDC2s.

• Even though the antagonistic effect of anti-IL-6R is diminishing 25 days after the last treatment, all treated STZ mice are protected from hyperglycemia.
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