Dissertation

The role of regulatory immune cells in autoimmunity and immune tolerance in type 1 diabetes

submitted by

Mag.rer.nat. Barbara PRIETL

For the Academic Degree of

Doctor of Philosophy

(PhD)

at the

Medical University of Graz

Department of Internal Medicine

Division of Endocrinology and Diabetology

under the Supervision of

Prof. Dr. Thomas PIEBER

2016

Statutory Declaration

“I hereby declare that this thesis is my original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”

Table of contents

Statutory Declaration 2

Table of contents 3

Abbreviations and Definitions 4

Abstracts 5

Introduction 7

Materials and Methods 20

Studies enclosed in this thesis 20

Study designs in detail 21

Immunological assessment 30

Results 39

Study compliance of healthy volunteers and patients 39

Differences in the percentage and function of peripheral immune cells between healthy controls, first degree relatives and T1D patients 39

Differences in the expression of GLP1-receptor in peripheral immune cells of healthy volunteers and T1D patients 55

Differences in the distribution of T cell subtypes throughout the human intestinal tract 60

Effects of cholecalciferol supplementation on the percentage of immune cell subtypes in the peripheral blood and intestinal tissue 65

Effects of liraglutide treatment on human immune cells *in vitro* and *in vivo* 82

Discussion 88

References 95

Abbreviations and Definitions

APC(s): antigen presenting cell(s)

DC: dendritic cell

DD: diabetes duration

FDR: First degree relatives

GADA: glutamic acid decarboxylase antibodies

GLP1-R: glucagon like peptide 1-receptor

FACS: fluorescence activated cell sorting

IA2: protein tyrosine phosphatase-related islet antigen 2

IL: interleukin

MMTT: mixed meal tolerance test

NK: natural killer cell

NKT: natural killer T cell

NOD: non obese diabetes mouse model

PBMC: peripheral blood mononuclear cell

Teff(s): effector (pro-inflammatory) T cell(s)

TGF-β: Transforming growth factor beta

Treg(s): regulatory T cell(s)

T1D: type 1 diabetes

T2D: type 2 diabetes

ZnT8: zinc transporter 8

Abstracts

The role of regulatory immune cells in autoimmunity and immune tolerance in type 1 diabetes

The immune-suppressive function of regulatory T cells (Tregs) is essential for maintaining immunological self-tolerance. The role of Tregs in autoimmune diseases, such as type 1 diabetes mellitus (T1D), is not fully understood yet. Reports from animal and in-vitro models suggest a pivotal role for Tregs in the pathogenesis of autoimmunity. In T1D treatment, vitamin D has been found to modulate Tregs and to correct auto-immune processes in animal models for T1D. Another treatment option could be based on glucagon-like peptide-1 (GLP1) which was recently described to interfere with inflammatory pathways involved in T1D. In five clinical studies performed within this thesis the role of Tregs and other peripheral immune cells in T1D was investigated and supplementation therapies, aiming to increase the percentage or the functional capacity of regulatory immune cells were performed. The characteristics of Tregs and other peripheral immune cells were mainly assessed by multi-parameter FACS analysis, quantitative real time PCR and ex-vivo co-cultures. The results of this thesis provide significant new information about the differences in peripheral immune-cell characteristics between healthy conditions and T1D and support the findings about the potential role for vitamin D therapy as a mechanism in controlling autoimmunity by restoring Tregs. Obtained data about the GLP1-receptor distribution on human peripheral immune-cells and the response of healthy participants to GLP1-receptor-agonist treatment provide a rationale for future trials, testing the complementary effect of vitamin D in combination with GLP1R-therapies in the context of T1D and other autoimmune diseases.

Die Rolle von regulatorischen Immunzellen in der Autoimmunität und Immuntoleranz in Typ 1 Diabetes

Die immune-suppressive Funktion von regulatorischen T-Zellen (Tregs) ist essentiell für die Aufrechterhaltung der immunologischen Selbst-Toleranz. Die genaue Rolle der Tregs in Autoimmunerkrankungen, wie Typ 1 Diabetes Mellitus, ist bisher nicht geklärt. Erkenntnisse aus Tier- und in-vitro Modellen suggerieren eine wesentliche Rolle von Tregs in der Pathogenese der Autoimmunität. In der Therapie von T1D zeigt Vitamin D wichtige Wirkungen auf Tregs und beeinflusst Autoimmun-Prozesse in T1D Tiermodellen positiv. Eine weitere Behandlungsoption könnte auf Glucagon-like Peptide-1 (GLP1) basieren, welches mit der Kontrolle von entzündlichen Prozessen in T1D in Zusammenhang gebracht wird. Im Rahmen dieser Thesis wurden fünf klinische Studien durchgeführt um die Rolle von Tregs und anderen peripheren Immunzellen in T1D zu untersuchen und die Auswirkungen von Therapien mit Vitamin D und GLP1R-Agonisten auf die Nummer und Funktion von Tregs zu erfassen. Die Merkmale von Tregs und anderen peripheren Immunzellen wurden hauptsächlich durch Multi-Parameter FACS Analyse, quantitative Real-Time-PCR und ex-vivo Kulturen untersucht. Die Ergebnisse dieser Thesis liefern signifikante neue Informationen zu den Unterschieden zwischen peripheren Immunzellen von gesunden Probanden verglichen mit Zellen von T1D-Patienten/innen. Weiters bestätigen die gewonnenen Daten die potentielle Rolle einer Therapie mit Vitamin D in der Kontrolle von Autoimmunität durch Interaktion mit Tregs. Die gewonnenen Daten zur Verteilung des GLP1-Rezeptors auf humanen peripheren Immunzellen und zur Antwort gesunder Probanden auf eine GLP1-Rezeptor-Agonisten Therapie liefern eine Rationale für zukünftige Studien, die den komplementären Effekt einer Kombinationstherapie mit Vitamin D und GLP1 im Kontext von Autoimmunität und T1D untersuchen sollten.

Introduction

The major tasks of the human immune system are the protection from pathogenic microorganisms and viruses as well as the identification and elimination of degenerated body cells. Immune answers need to be down-regulated after the completion of these tasks, otherwise harmful self-destruction of the bodies own tissue might develop. Many cellular and molecular pathways are involved in the regulation of immune answers and the maintenance of the immune homeostasis. Both, the protective as well as the auto-reactive immune responses are mediated by cells from the adaptive immune system, in particular lymphocytes named T- and B- cells. These subtypes of leukocytes are characterized by an intense effector activity, high antigen specificity, an enormous diversity in antigen recognition and a long-lasting memory function. The potency of B- and T-cells can cause serious damage to the bodies own tissues in the case of the failure of the immunological self-tolerance, which includes the recognition of self-antigens. The process of autoimmunity still is not fully understood, but it always includes the system of immune answers against the body´s own cells and tissues. Prominent examples for chronic autoimmune diseases are rheumatoid arthritis, where joints are targeted by auto reactive immune cells, diabetes mellitus type 1 (T1D), where pancreas cells are destroyed, celiac disease, where intestine cells are affected and Hashimoto´s thyroiditis where the thyroid gland cells are attacked.

Regulatory T cells, named Tregs, represent a distinct lineage of $CD4^{pos}$ T helper cells that play a central role in controlling immune responses and the maintenance of self-tolerance by active suppression of over- or self-reactive immune cells (1). First notes about T cells with a suppressive character were acquired from mouse and rat thymectomy models (2,3). In this models it was recognized that transfer of T cell suspensions depleted from Tregs from normal animals to syngeneic T-cell deficient athymic nude animals induced the spontaneous development of autoimmunity, such as T1D and inflammatory bowel disease. By contrast, the transfer of suspensions with enriched Treg populations induced the establishment of tolerance to organ transplants, prevented graft-versus-host disease after bone marrow transplantation and significantly decreased the susceptibility to allergies and autoimmunity (Fig. 1) (1).

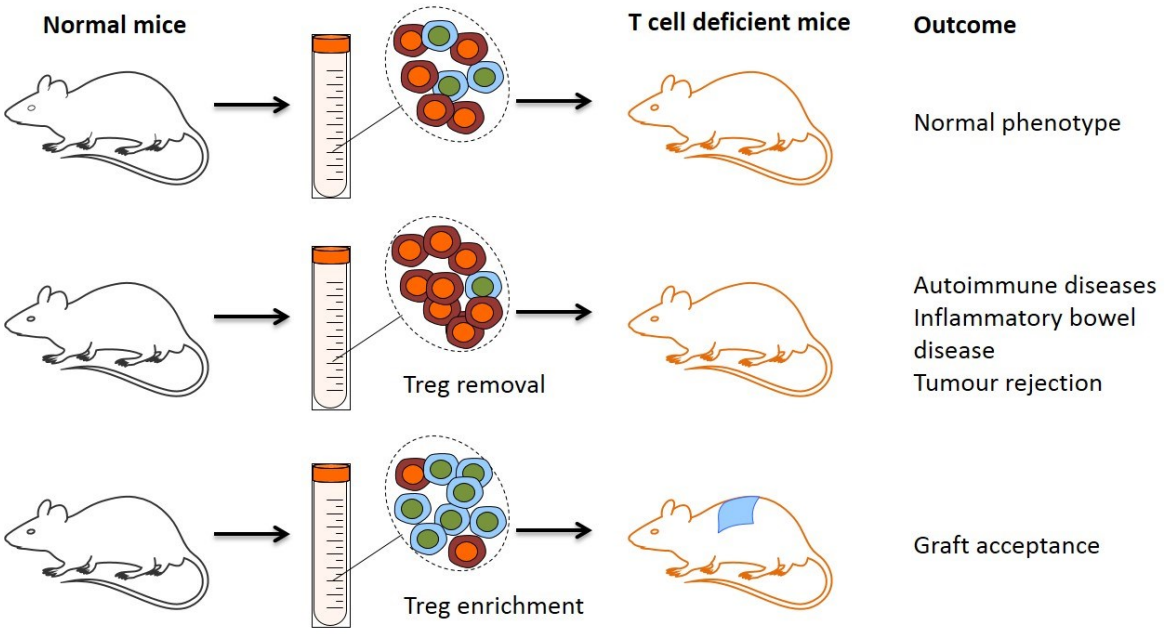

Figure 1: Effects of Treg deficiency or enrichment in mice. T cell suspensions prepared from normal mice can be depleted of CD25+CD4+ regulatory T cells (Treg) and transferred to syngeneic T cell-deficient mice (such as athymic nude mice). The recipient mice spontaneously develop autoimmune diseases and inflammatory bowel disease and reject tumor cells. By contrast, when Tregs are enriched from normal mice and transferred, the recipient nude mice accept allogeneic skin grafts. Adapted from Sakaguchi et al. (1) with permission from Cell.

Subsequently, Tregs were also identified in the peripheral blood of humans and described to play a major role in the pathophysiology of autoimmune diseases (reviewed by Baecher-Allan (4) and Buckner (5)). Today the most accurate and common definition of markers to identify human Tregs is the parallel expression of the CD4 receptor, the high expression levels of interleukin-2 receptor (CD25), low expression levels of interleukin-7 receptor (CD127) and the positive signals for the forkhead box protein 3 (FOXP3) transcription factor, although many other surface proteins might play a major role for suppressive function of Tregs (reviewed by Schmetterer (6)).

FoxP3 is known to be the master regulator of Treg development and functionality (7). In humans, FoxP3 gene mutations are linked to the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterized as a multiorgan autoimmune syndrome with an early-onset and a bad prognosis (reviewed by Ochs, 2005 (8)). Fundamental animal studies about the role of FoxP3 in Treg development and function were mainly performed using the rodent model of IPEX, the scurfy mouse (9–11).

In both, mice and humans, Tregs can develop either in the thymus or be induced from lymphocytes in the periphery by conversion of effector T cells into regulatory FoxP3 expressing Tregs. Especially dendritic cells are found to play a role in the peripheral induction of Tregs (12,13) . This process is known to depend on co-factors, such as interleukin-10 (IL-10) and TGF-β. Once matured, the function and activity of Tregs depends on the activation of the T-cell receptor/CD3 complex, similar as found in effector T cells. After activation, the regulatory effect of Tregs is antigen non-specific and not restricted to effector T cells (3,14). Tregs can actively block immune responses, inflammation and tissue destruction by direct mechanisms, including cell-cell contact, cytolysis, interleukin-2 (IL-2) deprivation and changes in the cytokine milieu (Fig. 2). Additionally, the indirect suppression of immune cell responses via the modulation of antigen-presenting cells (APCs), such as dendritic cells (DC), is a known mechanism of Tregs suppressive activity. This includes the inhibition of DC maturation (15) and down regulation of CD80/86 receptor expression (16,17) as well as the induction of the regulatory enzyme IDO (enzyme indoleamine 2,3-dioxygenase) in dendritic cells (18) (Fig.2).

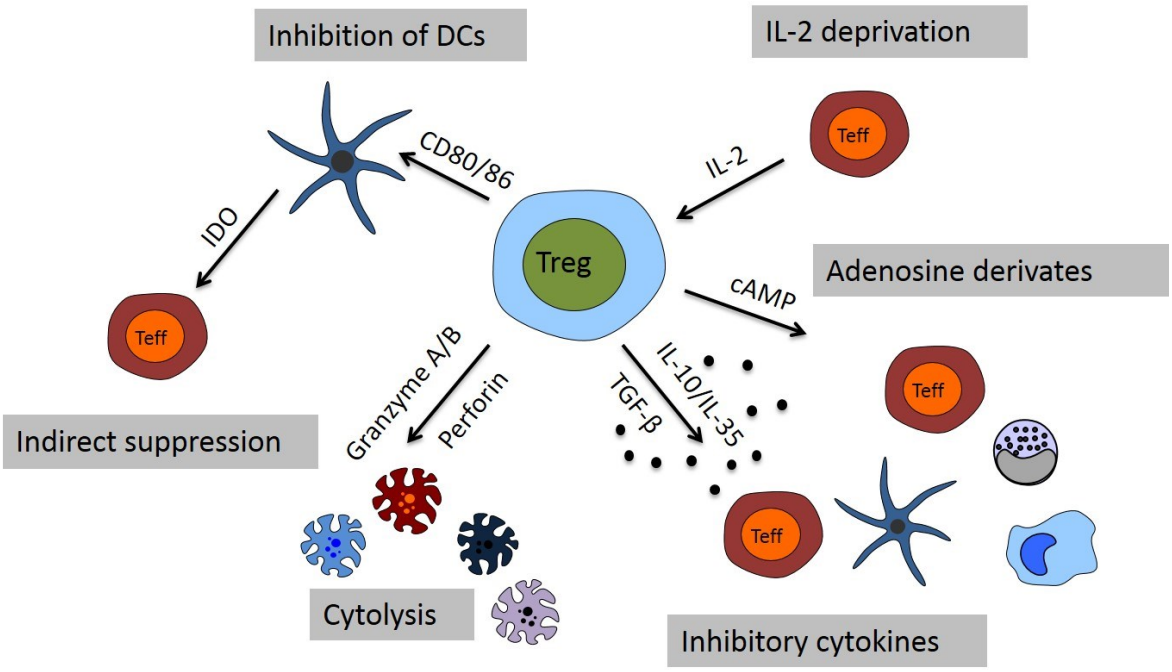

Figure 2: Best known mechanisms of Treg-mediated immune cell suppression. Tregs can regulate the activation of other immune cells by a variety of molecular mechanisms, which might work in a complementary way. Tregs can target antigen presenting cells (APCs), such as dendritic cells (DCs), which leads to a modulation of the co-stimulatory receptors CD80/86. This causes a decreased signaling for T cell activation and leads to an indirect suppression of T effector cells (Teffs). Additionally, Tregs can cause an increased production of indoleamine 2,3-dioxygenase (IDO) in DCs which also decreases Teff activation. Another described mechanism includes the competition for interleukin 2 (IL-2), which plays a critical role in the proliferation and activation of Teffs. Tregs can produce inhibitory adenosine derivates, such as cyclic adenosine monophosphate (cAMP) or inhibitory cytokines, such as IL-10, IL-35 and TGF-β. All this inhibitory molecules can down-regulate the function of the target cells. Finally, Tregs can also suppress target cells by the direct induction of cytolysis, catalyzed by secreted Granzyme B and Perforin.

In healthy conditions, the percentage of Tregs within $CD4^{pos}$ T cells is about 5%. Despite the early observations that peripheral $CD4^{pos}CD25^{pos}$ Tregs are reduced in autoimmune diseases, such as T1D (19), it is now clear that the percentage of Tregs in the peripheral blood of patients suffering from T1D is similar to that of healthy controls (20–23). Nevertheless certain subsets of Tregs might be decreased early after the disease onset (24). Such contradictory results about the percentage of peripheral Tregs might be caused by heterogeneity within the Tregs population and a lack of consensus regarding the best combination of markers to identify and quantify Tregs (6). Many studies investigating autoimmune diseases reported defects in the function of Tregs, from both mice models and human patients (reviewed by Buckner (25)). For example, in T1D an altered function of Tregs has been described by assessing CD4+CD25+ T cells (21). Additionally to the impaired function of Tregs, a resistance of effector T cells towards the Treg

mediated suppression could contribute to the development of autoimmunity (26,27).

The functional failure of Tregs might play a major role in the pathogenesis and prognosis in type 1 diabetes mellitus, where cytotoxic and helper T cells trigger a destruction of pancreatic, insulin-producing β-cells. According to this, T1D is classified as a T cell mediated type IV hypersensitivity, including a major role for both disturbed T helper- and cytotoxic-T cell-responses. In the process of autoimmune reactions macrophages with a high destructive potential are activated, mostly by interferon-γ produced by Th1 cells. Subsequently, cytotoxic $CD8^{pos}$ T cells as well as Th2-cells are activated. Interleukin-4 secretion by activated Th2 cells can attract eosinophilic neutrophils to attack insulin producing β-cells in the pancreatic tissue. Additionally the direct attack of β-cells by $CD8^{pos}$ T cells is mediated by the induction of CD95 (Fas) molecules on the surface of pancreatic cells after exposure to interleukin 1 and nitric oxide, which is mostly produced by activated macrophages. In addition, dendritic cells can clear up after the β-cell destruction and present the β-cell antigens to T-cells in a way how normally pathogenic, non-self-antigens are presented. This leads to a continuing activation of T cells and other immune cells and an induction of the secretion of autoantibodies by B cells. Autoantibodies to islet and β-cell antigens, are present in 70-80% of newly diagnosed patients, but can often be detected long before the clinical disease onset. The most reliable and best characterized autoantibodies are islet cell autoantibodies (ICAs), autoantibodies to glutamic acid decarboxylase (GADAs), insulin autoantibodies (IAAs), and autoantibodies to transmembrane tyrosine phosphatase (IA2As) and against the ZnT8 molecule (ZnT8As). In large clinical trials it has been verified that the risk to develop T1D increases with the grade of co-existence of different autoantibodies, including the highest risk for those individuals having four autoantibodies in combination (28).

The well-established model for the pathogenesis of T1D suggests that genetically susceptible individuals are exposed to an unknown environmental trigger which induces auto reactivity of immune cells towards pancreatic β-cells. During this process, autoantibodies arise in the circulation, but clinical symptoms do not appear until more than 80% of the β-cells are destroyed. Recently this model has been modified by the notion that pancreatic β-cells may persist and never reach zero in some T1D patients and that the viability of β-cells might be related to

factors like age, BMI and physical activity. Nowadays, T1D is seen as a relapsing-remitting autoimmune disease (reviewed by Atkinson, 2012 (28)), which is most often diagnosed during childhood and adolescence. In adults, T1D can be seen in the classical form but also as latent autoimmune disease of adults (LADA) form of diabetes (reviewed by Leslie et al, 2008 (29)). In both, children and adults, the incidence of T1D seems to vary according to seasonal changes, showing a higher peak in autumn and winter months and a lower peak in the summer period (30). Interestingly, the incidence rates of T1D are very different among the reporting countries worldwide, for example T1D is very uncommon in India and China where the incidence rate is 0.1/100000 per year, whereas the incidence rate for T1D in Finland is 60 cases/ 100000 per year. With a few exceptions, the incidence rate for T1D is particularly high in northern countries in Europe and North America. In the last years, large epidemiological studies proved that the incidence of T1D is increasing worldwide and a trend towards earlier manifestation has been noticed, showing the highest increases in the incidence rates in children beyond 5 years of age (31,32).

At time of clinical manifestation, the classical symptoms caused by chronic insulin deficiency and resulting hyperglycemia in the blood and the urine, are polydipsia, polyuria, polyphagia but concurrent weight loss. Acute complications in T1D include diabetic ketoacidosis and hyperglycemic coma. This requires an immediate need for exogenous insulin replacement and finally leads to a life-long daily insulin injection therapy. Besides the classical symptoms T1D can induce several severe complications, mainly at the vascular level in organs such as eyes, kidneys and the foot (33).

So far, it remains unclear what induces the autoimmune responses to the pancreatic, insulin-producing β-cells. Many theories have been developed, suggesting a role of viral infections, sensitivity of β-cells to free radical or cytokine-induced damage, molecular mimicry with environmental agents, breakdown of central tolerance, defects in dendritic cell trafficking, alteration of self-antigens, defective MHC expression on immune cells and finally defects in the peripheral tolerance mechanisms (28). This supports the picture about T1D as a complex and multifactorial disease, which cannot be explained by a clear pattern of inheritance (Fig.3). In fact, approximately 85% of newly diagnosed cases affect patients with no family history of T1D. However, there are genes that might play a

role in the pathogenesis of T1D. The strongest genetic association comes from the allelic variations in the human leukocyte antigen complex (HLA). Class II HLA genes, which seem to have the highest impact, encode for proteins involved in the antigen presentation and a great proportion of T1D patients carry the HLA-DR3 or HLA-DR4 class II antigen. Besides the HLA genes, polymorphisms in the promoter region of the insulin gene seem to have high associations with T1D, but also genes encoding for CTLA-4 (cytotoxic T lymphocyte associated-4), CD25 (interleukin 2 receptor) and PTPN22 (protein tyrosine phosphatase) might be associated with T1D (34). Notably, the majority of the genes that are thought to contribute to the pathogenesis of T1D encode molecules that are related to immune responsiveness and immune regulation.

Besides genetic susceptibility, environmental factors might play a critical role in disease development. This theory is supported by the described variance in incidence rates according to the geographic location and the observations showing a rapid assimilation of the local incidence rates when individuals change from a low- to a high-incidence country. There are many different studies supporting a role of various environmental factors in T1D, but often the results of the studies are controversial (all reviewed by Atkinson, 2012 (28)). For example, the hygiene hypothesis, assigns a role for the decreased or altered exposure of the immune system to common pathogens in the pathogenesis of T1D and allergies. Another thesis blames the increase in childhood obesity, causing excessive demand of insulin production by pancreatic β-cells and the resulting stress-induced β-cell damage, described as overload hypothesis. Another hypothesis correlates the altered gut microbiota and increased gut permeability as result of changes in diet with the increased incidence for T1D. Viral infections, particularly enterovirus infections, are also thought to play a role in the pathogenesis of T1D just as well as perinatal factors, such as pre-clampsia, neonatal infections and Caesarian section. Additionally, diet is caused to play a role in T1D, such as breast feeding versus early cow-milk exposure, early feeding of gluten and cereal components, low vitamin D levels and nitrosamine compounds.

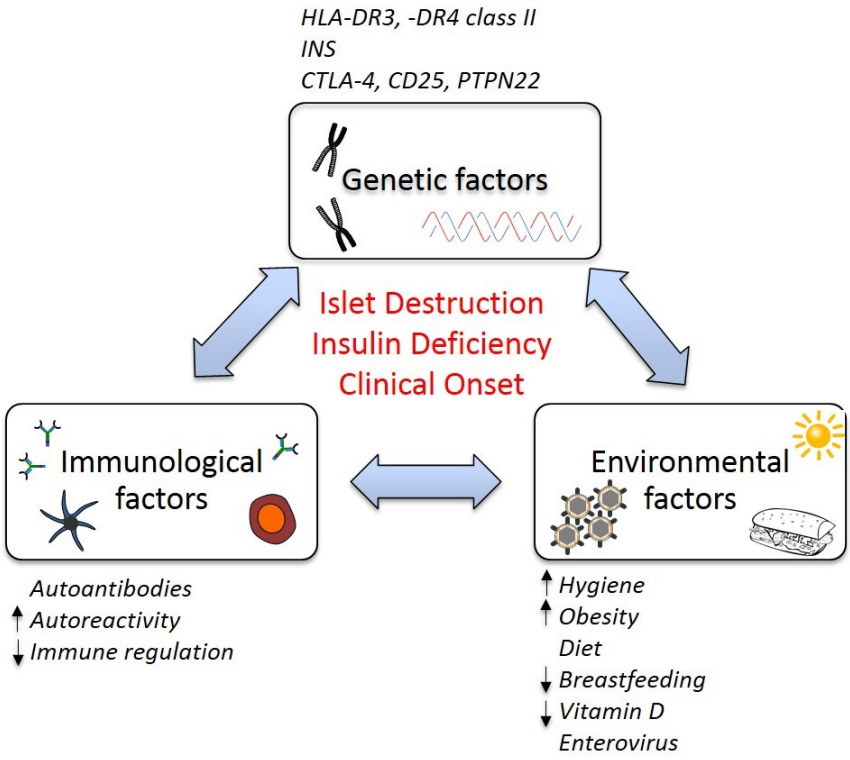

Figure 3: Multiple causes thought to be involved in the development and pathophysiology of type 1 diabetes (T1D). Prenatal genetic factors such as distinct human leukocyte antigen (HLA) variants, are suspected to play a role in the pathophysiology of type 1 diabetes (T1D). Apart from genetic susceptibility, environmental factors such as increased hygiene, altered diet, increased obesity and less breastfeeding seem to play a role in this autoimmune process. But also altered lifestyle, causing less endogenous vitamin D production by decreased exposure to sunlight are suspected to have an impact on T1D development. Another hypothesis accuses enteroviral infections to trigger the onset of T1D. Probably, a combination of all this factors leads to the onset of auto-reactivity, including production of auto-antibodies by B-cells, activation of self-reactive T-cell clones and decreased immune regulation. The subsequent destruction of insulin-producing pancreatic β-cells finally leads to insulin deficiency and the clinical onset of type 1 diabetes.

So far, no therapy can decrease or stop autoimmune destruction of pancreatic β-cells without causing severe side effects (35). Strategies to increase Treg numbers or to enhance stable Treg function *in vivo* have gained attractiveness in the last years facing new therapies for T1D. Adoptive transfer of *in vitro* expanded human Tregs and compounds like rapamycin, anti-CD3 antibodies, and IL-2-anti-IL-2 complexes have been used to re-establish immune homeostasis in T1D based on Tregs (36–39), but so far failed as serious T1D therapy due to causing mayor side effects.

One immune modulatory drug, namely vitamin D, has emerged as safe supportive treatment for diseases caused by impaired immune cell homeostasis (40). The inactive form of vitamin D, named cholecalciferol, can be synthesized in the

human skin from 7-dihydrocholesterol when exposed to UVB radiation or can be obtained by diet. Endogenous produced cholecalciferol is immediately bound by vitamin D binding proteins (DBP) or albumin and translocated to the circulation, whereas ingested cholecalciferol is first transported to the circulation embedded in chylomicrons and then binds to DBP or albumin. In the liver cholecalciferol is hydroxylated, catalyzed by the four hepatic cytochrome P-450 enzymes (CYP2R1, CYP2J2, CYP3A4 and CYP27A1). This results in the production of the inactive form calcidiol (25-hydroxyvitamin D; 25(OH)D) which represents the major circulating vitamin D metabolite and is the most validated serum parameter to define the human vitamin D status (41). Subsequently, calcidiol is converted by the kidney enzyme CYP27B1 to form the biologically active compound calcitriol ($1,25(OH)_2D$) (Fig.4). Serum calcitriol levels are regulated in a renal negative feedback loop facilitated by the inhibition of CYP27B1 by high levels of calcitriol and activation of the enzyme CYP24A1 (24-hydroxylase), which metabolizes calcitriol into the inactive, water soluble form, calcitroic acid. Additionally, CYP27B1 is under the tight control of the parathyroid hormone and the phosphaturic hormone fibroblast growth factor 23 (FGF-23). The conversion of calcidiol into the active form of vitamin D can also occur extra-renal in multiple tissues, such as muscles, colon, prostate, pancreas and the immune system. All these tissue cells express CYP27B1 and are able to catalyze the conversion in an autocrine or paracrine manner. Especially the production of calcitriol by immune cells, such as macrophages and dendritic cells, can lead to a high local concentration of the active form of vitamin D because of a lack of feedback mechanisms compared to kidney cells (reviewed by Baeke (42)).

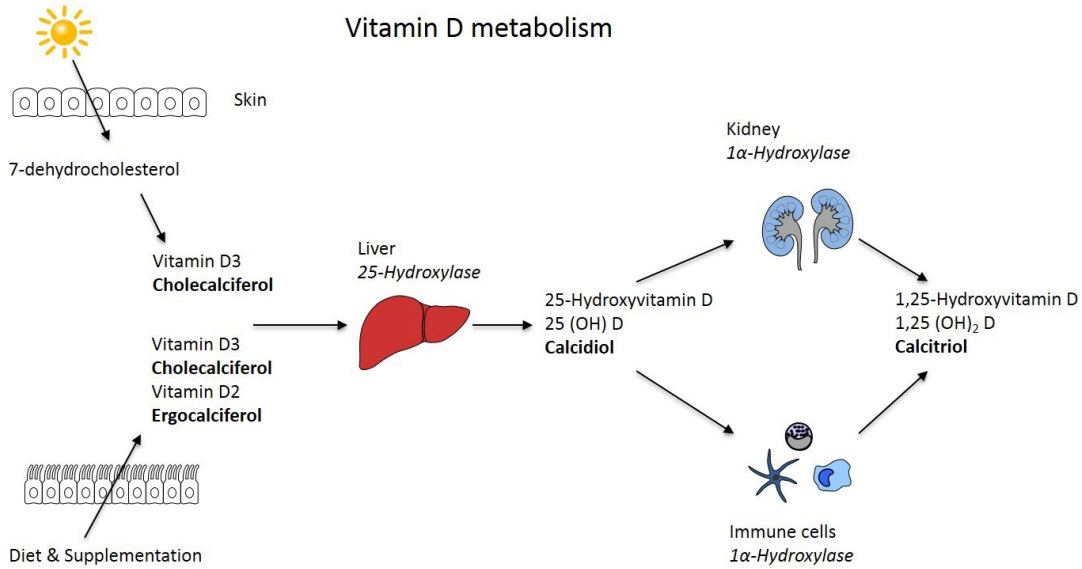

Figure 4: Schematic overview for the metabolism of vitamin D in the human system. Synthesis of cholecalciferol occurs in the skin in response to UVB exposure or is directly obtained from dietary components and supplementation. In the circulation cholecalciferol is bound by vitamin D-binding proteins (DBP) and transported to the liver where a conversion into calcidiol happens. This main storage form of vitamin D can then be converted by kidney cells, immune cells and other distinct tissue cells, such as prostate cells (not shown in the graph), generating the active hormone calcitriol.

The active form of vitamin D ($1,25(OH)_2D$, calcitriol) helps to suppress pro-inflammatory Th1 and Th17 responses, while promoting the Th2 and regulatory T cell phenotype by enhancing the production of interleukin IL-4, IL-5 and IL-10 (43–45). Calcitriol supplementation has also been shown to influence proliferation, differentiation and various functions such as cytokine production by antigen presenting and adaptive immune cells (46) and it reduces the number of T cells that are resistant to apoptosis in non-obese diabetic (NOD) mice (45). In humans, vitamin D might also control the activation of T cells and the T cell receptor signaling pathways (47) (Fig. 5).

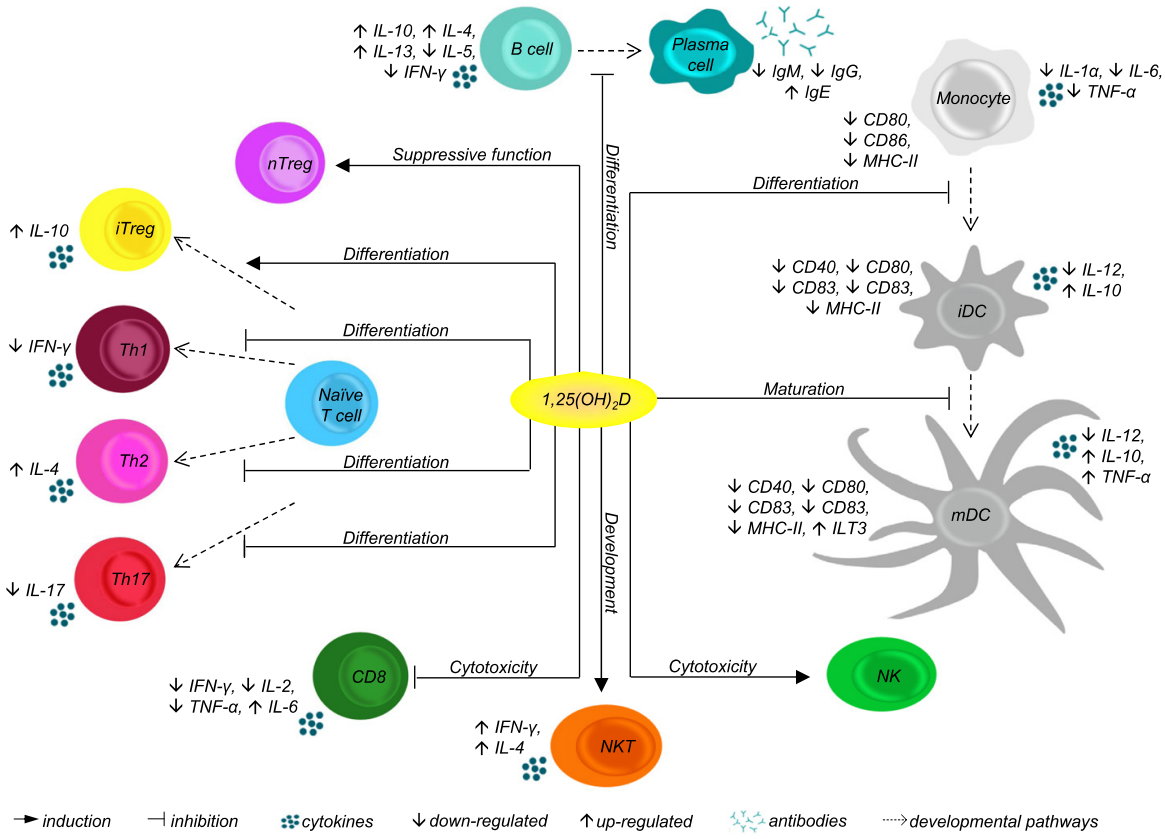

Figure 5: The role of vitamin D in the activation and regulation of innate and adaptive immune cells. This overview shows the main effects of the active form of vitamin D, calcitriol ($1,25(OH)_2D$), on monocytes, dendritic cells (DC), natural killer cells (NK), natural killer T cells (NKT), T-cell subtypes and B cells. Adapted from Peelen et al.(48) With permission from Autoimmunity Reviews.

Studies using the inactive form of vitamin D succeeded to correct immunological defects in diabetes-prone mice and to reduce the diabetes development in mice when using an early and life-long high dose vitamin D3 (cholecalciferol) supplementation (49). The positive effects of different forms of vitamin D on the immune system have been demonstrated in various animal models, but data from human studies are scarce. Large cohort studies showed that the risk to develop T1D is linked to vitamin D deficiency (50,51) and that vitamin D supplementation during infancy can protect against T1D (52–54). However, in human studies treatment with calcitriol did not reduce loss of ß-cell function in new onset T1D (55,56), but in a subtype of T1D (LADA) the ability to preserve ß-cell function was reported (57). The use of calcitriol or analogues to supplement humans is restricted to a lower dosage due to the potential toxic side effects such as hypercalcemia. Using a high-dose supplementation of the inactive form of vitamin

D (cholecalciferol) has been described as safe in humans (58) and a 12 months supplementation succeeded in significantly increasing the level of circulating Tregs in newly diagnosed T1D patients (59). In healthy volunteers we could recently show a significant increase of peripheral Tregs after supplementation with high doses of cholecalciferol for 8 weeks (60).

Another medication, that recently emerged in the field of autoimmunity are incretin based treatments with GLP-1 (glucagon like peptide-1) analogues or DPP-4 (di-peptidyl-peptitase-4) inhibitors. Glucagon like peptide-1 is an important gastrointestinal incretin hormone which is secreted by endocrine L-cells in the ileum and colon within minutes after food intake and which plays a major role in the postprandial insulin secretion. Besides stimulation of the insulin release it also decreases the glucagon secretion, inhibits gastric emptying, reduces appetite and food intake, induces beta-cell proliferation and enhances beta cell resistance to apoptosis (61,62) (Fig.6).

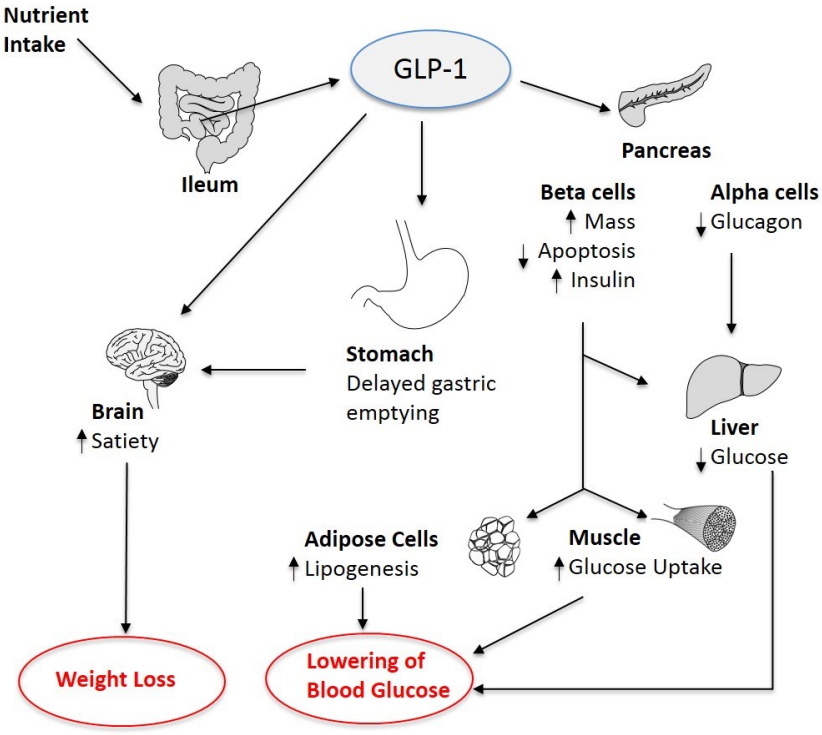

Figure 6: The pleiotropic effects of glucagon-like-peptide-1 (GLP1) in humans. GLP-1 is secreted by L-cells in the ileum as response to food intake and has direct effects on the brain, the stomach and the α- and β-cells of the pancreas. Together with subsequent effects on the adipose tissue, the muscles and the liver it causes weight loos and lowers blood glucose levels. Adapted from Bradley et al., 2010 (63) with permission from Nutrition.

GLP-1 is rapidly metabolized by DPP-4. Synthetic GLP1-analogues, such as exenatide or liraglutide, have a prolonged half-life whereas DPP-4 inhibitors decrease the normal degradation of GLP-1. Both drugs have recently been approved for the treatment of type 2 diabetes mellitus (T2D). In T2D GLP-1 analogues and DPP-4 inhibitors are used to influence the glucose homeostasis, but there is growing evidence that they also have beneficial effects on body weight, blood pressure and immunomodulation. Liraglutide, a GLP-1 analogue, selectively activates the GLP-1 receptor (GLP-1R), a Gs protein–coupled receptor (64). The expression of GLP-1R has been described in many primate tissues, such as pancreas, lung, intestine, kidney and heart (65). Recent studies suggest immune-modulatory properties of incretin based treatments like GLP- 1 analogues or DPP- 4 inhibitors (66–69) but the majority of these studies focus on cell culture or animal models. Recently, a direct effect of liraglutide treatment on the number and stimulation of iNKT cells in T2D patients suffering from psoriasis has been described (68). Furthermore, liraglutide treatment can reduce inflammatory cytokines produced by innate immune cells in T2D patients (70). In a placebo controlled study, treatment of T2D patients with DPP-4 inhibitors for 12 weeks significantly reduced pro-inflammatory cytokines, kinases and chemokines in the circulation (69). Additionally, studies with animal models for T1D showed disease remission and enhanced numbers of regulatory T cells due to treatment with GLP-1R agonists (71,72).

The aim of this thesis was to investigate the role of human regulatory and pro-inflammatory immune cells in the pathogenesis of T1D. Differences in the composition of regulatory and pro-inflammatory immune cell subtypes were investigated in a big cohort study, including samples from healthy controls, T1D patients and first-degree relatives of the involved patients. Supplementation therapies, aiming to increase the percentage or the functional capacity of regulatory immune cells were investigated in 4 clinical studies investigating the effect of high-dose vitamin D supplementation on peripheral blood cells from healthy controls and newly-diagnosed T1D patients as well as on the gut associated immune cells from healthy volunteers. Additionally, the effects of liraglutide and DPP4-inhibitor treatment on peripheral immune cells were studied *in vitro* and in a clinical trial including adult healthy volunteers.

Materials and Methods

Studies enclosed in this thesis

Five different clinical studies are enclosed in this thesis. An overview concerning the study populations, study designs, interventions and obtained samples for analysis is given in table 1. For all studies ethical approval was obtained from the local Ethics Committee of the Medical University of Graz, Austria and written informed consent was provided by healthy participants and patients with T1D or their guardians before any study-related activities. All studies were performed in accordance with Good Clinical Practice and the Declaration of Helsinki.

Study ID	**Healthy VitD**	**ADPP001**	**ADPP002**	**ADPP003**	**ADPP004**
Participants					
- groups	H	H / T1D / FDR	T1D	H	H
- n	59	373	30	16	15
- age					
- DD	--	mixed	< 3 months	--	--
Study design	randomized, double-blinded	cohort study, cross-sectional	randomized, double-blinded	pilot study	randomized, blinded for laboratory
Intervention					
- drugs	cholecalciferol	--	cholecalciferol	cholecalciferol	Liraglutide
	placebo	--	placebo	--	Saxagliptin
- dosage	140 000 IU/m (≈ 70 IU/kg/d)	--	140 IU/kg/d + 70 IU/kg/d	140 IU/kg/d + 70 IU/kg/d	Liraglutide: 0.6 mg/d + 1.2 mg/d Saxagliptin: 5 mg/d
- duration	12 weeks	--	12 months	8 weeks	4 weeks

Table 1: Overview for the studies included in this thesis. Study IDs are given as described in the text. Abbreviations for the different patient groups: H: Healthy participants, FDR: First Degree Relatives, T1D: Type 1 Diabetes Mellitus patients. Age is given in mean ± SD. n = number of all participants involved in the study. DD: Diabetes Duration, ADPP: Austrian Diabetes Prevention Program.

Study designs in detail

Healthy vitamin D intervention study

A randomized, double-blinded, placebo controlled study was performed including healthy volunteers and registered at ClinicalTrials.gov (NCT01248442). Healthy participants aged between 18 and 65 years were recruited from the outpatient clinic in Graz, Austria. Exclusion criteria were preexisting hypercalcemia, any inflammatory systemic disease, disorders in calcium metabolism, kidney diseases, and a family history in autoimmune diseases, allergic predisposition and pregnancy. All individuals were randomized into two study groups (vitD group and placebo group) using the Randomizer program (http://www.randomizer.at) and received 3 monthly doses of either a cholecalciferol supplementation (140000 IU of Oleovit D3 per month, Fresenius Kabi, Austria) or equal amounts of placebo (almond oil) for 12 weeks. Study compliance was assessed by the performance of regular phone contacts. In total 4 visits were performed within 12 weeks for each study participant. After the baseline visit, patients were seen after 4, 8 and 12 weeks. Safety evaluation, study medication supply and venous blood draw were performed at each visit after a 12h overnight fast. At baseline and after 12 weeks a total volume of 126 ml blood was drawn, after 4 and 8 weeks a total of 16 ml venous blood was drawn. Demographic baseline data are shown in the result section (Tab. 11). An overview about study compliance and exclusion of participants is given in an adapted CONSORT 2010 flow diagram (Fig. 7).

ADPP001

The “Austrian Diabetes Prevention Program 001” study was designed as a cohort study recruiting T1D patients with different disease durations, healthy controls and first-degree relatives of T1D patients (mainly children and siblings of patients). All participants had to perform one single visit after a 12h overnight fast. Adults and adolescent volunteers above the age of 14 years donated 85 ml blood, children with an age between 6-14 years where asked to donate 22 ml of blood in total. Study participants were also asked to perform a mixed meal tolerance test (MMTT) in order to assess the residual pancreatic beta cell function. Study participants aged at least 6

years were recruited from the diabetes outpatient clinics in Graz, Vienna and Salzburg, Austria. Exclusion criteria were pregnancy and a chronic disease, except T1D. Demographic data are shown in the results section (Tab. 4).

ADPP002

The ADPP002 study was performed as a randomized, double-blinded and placebo controlled study and registered at ClinicalTrials.gov (NCT01390480). Newly diagnosed T1D patients, with a diabetes duration less than 3 month (definition according to ADA criteria (73)), were recruited from the diabetes outpatient clinics in Graz, Vienna and Salzburg, Austria. Patients were aged at least 6 years and not older than 60 years. Exclusion criteria were preexisting hypercalcemia, any inflammatory systemic or kidney diseases, disorders in calcium metabolism and pregnancy. Individuals were randomized into two study groups (vitD group and placebo group) using the Randomizer program (http://www.randomizer.at) and subsequently received a weekly oral cholecalciferol supplementation (70IU/ kg/ day of Oleovit D3, Fresenius Kabi, Austria) or equal amounts of placebo (vegetable oil) for 12 months including an additional loading dose (140 IU/kg/day) for the first month. This initial doubling of the dose was performed in order to achieve vitamin D levels in the upper normal range in all study participants within a short period of time. Study compliance was assessed by the use of a study diary for each patient and regular phone contacts. In total 7 visits were performed within 13 months for each study participant. After the baseline visit, patients were seen after 1, 3, 6, 9, 12 and 13 months. Safety evaluation, study medication supply and insulin dose adjustments were performed at each visit. HbA1c measurement and blood donation for the analysis of immunological parameters was only performed at baseline and after 3, 6 and 12 months. A mixed meal tolerance test (MMTT) was performed at baseline, after 6 and 12 months to assess the residual pancreatic beta cell function. For all laboratory measurements venous blood was drawn after a 12h overnight fast. Volumes per blood draw per visit are summarized in table 2. Demographic baseline data are shown in the result section (Tab. 11). An overview about study compliance and exclusion of participants is given in an adapted CONSORT 2010 flow diagram (Fig. 8).

	6-9 years		10-13 years		> 14 years	
Visits	**Blood draw**	**Leuko abs**	**Blood draw**	**Leuko abs**	**Blood draw**	**Leuko abs**
Baseline	22 ml	6.4 (5.3 – 7.8)	22 ml	7.8 (5.5 – 9.5)	85 ml	6.3 (5.4 – 7.6)
1 month	3 ml	6.9 (6.3 - 10)	3 ml	6.8 (5.5 – 9.3)	16 ml	7 (5.7 – 8.3)
3 months	3 ml	6.6 (4.7 - 7.9)	19 ml	6.7 (5.9 - 8.8)	81 ml	6.7 (4.9 – 9)
6 months	22 ml	10 (5.8 - 12)	22 ml	8.7 (6.9 – 9.8)	85 ml	6 (5.3 – 8.5)
9 months	10 µl	--	10 µl	--	16 ml	7.8 (5.6 – 9.2)
12 months	22 ml	7.9 (7 – 9.2)	22 ml	7.4 (7.3 – 9.4)	85 ml	7.8 (6.4 – 11)
Follow up	--	--	--	--	16 ml	7.9 (7.1 – 9.6)

Table 2: Blood volume per draw and absolute number of leukocytes (x 10^6) per ml blood (given in median + IQR) for participants enrolled in the ADPP002 study.

ADPP003

The ADPP003 study was designed as an open pilot study including high-dose vitamin D intervention and gastrointestinal biopsy sampling in healthy volunteers and registered at ClinicalTrials.gov (NCT01538485). Participants aged between 18 and 40 years were recruited from the outpatient clinic in Graz, Austria. Exclusion criteria were preexisting hypercalcemia, any inflammatory systemic disease, disorders in calcium metabolism, kidney diseases, a family history in autoimmune diseases, allergic predisposition, a body mass index not meeting the range between 20-30 kg / m^2, smoking and pregnancy. All individuals received an initial loading dose of oral cholecalciferol supplementation (140 IU/kg/day of Oleovit D3, Fresenius Kabi, Austria) for 4 weeks in order to achieve vitamin D levels in the upper normal range. This phase was followed by a weekly oral cholecalciferol supplementation (70IU/ kg/ day) for 4 weeks. A follow up visit was performed after the total study duration of 10 weeks. In total 7 visits were performed within 10 weeks for each study participant. At baseline study participants underwent a gastro duodenoscopy followed by a colonoscopy on the next day. At gastro duodenoscopy, biopsies were drawn from the gastric corpus (GC), the gastric antrum (GA) and the descending part of the duodenum (DD). At colonoscopy, biopsies were drawn from the terminal ileum (TI),

the appendiceal orifice region (AO), the ascending colon (AC) and the sigmoid colon (SC) (Fig. 32). In order to prepare the gastrointestinal tissue for gastro duodenoscopy and colonoscopy all volunteers received a polyethylene glycol-based electrolyte solution (MOVIPREP) based laxative. Six biopsies were taken per region. Two out of six biopsies were immediately fixed with formalin for pathological assessment. Two biopsies were put into RNAlater (Sigma Aldrich, Germany) and used for RNA extraction and the remaining two samples were immediately put into complete RPMI media containing 10% fetal calf serum (c-RPMI-10), glutamine and penicillin/streptomycin (all from Life Technologies, Germany) and used for the isolation of lamina propria mononuclear cells. Gastro duodenoscopy and colonoscopy were repeated after 8 weeks of cholecalciferol supplementation. Safety evaluation was performed at each visit, study medication supply and venous blood draw were performed at all visits after a 12h overnight fast. At baseline, after 8 weeks and after 10 weeks 44 ml blood were drawn. At the safety visit, performed 4 weeks after the start of the supplementation 8 ml blood was drawn in total. Demographic baseline data are shown in the result section (Tab. 8). An overview about study compliance and exclusion of participants is given in an adapted CONSORT 2010 flow diagram (Fig. 9).

ADPP004

The aim of the Austrian Diabetes Prevention Program 4 study was to assess the short term effects of the treatment with GLP-1R agonists or DPP4-inhibitors on peripheral immune cells in healthy volunteers. The study was designed as monocentric, blinded and randomized. Healthy adults, aged at least 18 years were enrolled and the study was registered at ClinicalTrials.gov (NCT01782261). Exclusion criteria were preexisting inflammatory systemic diseases, disorders in calcium metabolism, kidney diseases, a family history in autoimmune diseases, allergic predisposition and pregnancy. All individuals were randomized into two study groups (liraglutide and saxagliptin group) using the Randomizer program (http://www.randomizer.at). Study medication was either liraglutide (Victoza®, Novo Nordisk, Denmark) to be applied as subcutaneous self-injection by the study participant or saxagliptin (Onglyza®, BMS, USA) to be administered orally. Liraglutide was initially applied in a starting dose (0.6 mg/day) for 1 week followed by

self-injection of 1.2 mg/day for 3 weeks. Saxagliptin was taken orally in a constant dose (5 mg/day) for the whole duration of 4 weeks.

Study compliance was assessed by the performance of regular phone contacts. In total 4 visits were performed within 8 weeks for each study participant. After the baseline visit, patients were seen after 2, 4 and 8 weeks. Safety evaluation, study medication supply and venous blood draw were performed at each visit after a 12h overnight fast. In total 65 ml of blood were drawn per visit. Demographic baseline data are shown in the result section (Tab. 12). An overview about study compliance and exclusion of participants is given in an adapted CONSORT 2010 flow diagram (Fig. 10).

Adapted CONSORT 2010 Flow Diagram

Study ID: **Healthy VitD**

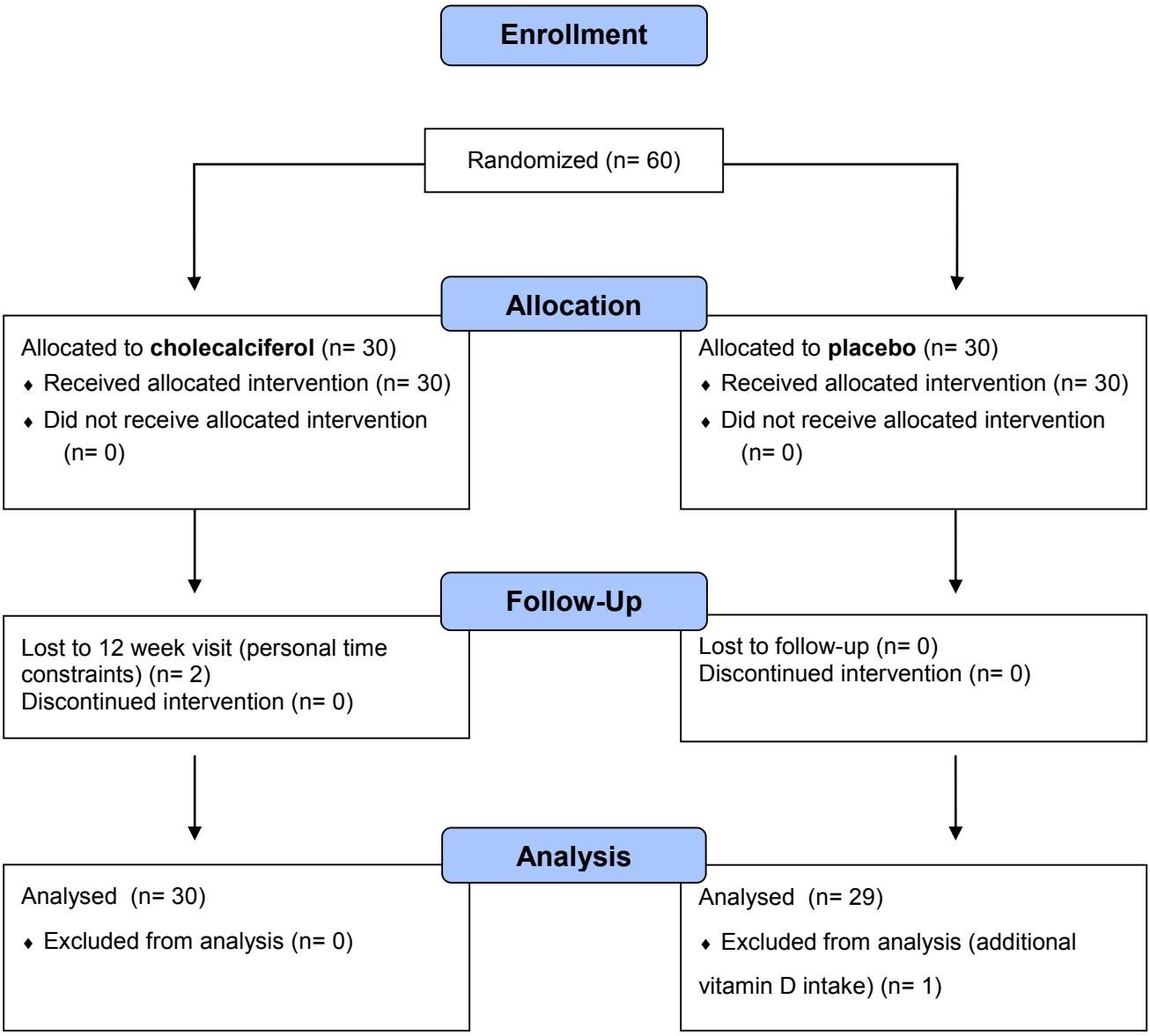

Figure 7: Adapted Consort Flow Diagram for the Healthy Vitamin D study
This diagram summarizes the progress through the different phases of the randomized, double blinded, placebo controlled trial. This includes enrollment, intervention allocation, follow-up phase and data analysis. In this RCT healthy adult participants were included and it was registered at ClinicalTrials.gov (NCT01248442).

Adapted CONSORT 2010 Flow Diagram

Study ID: **ADPP002**

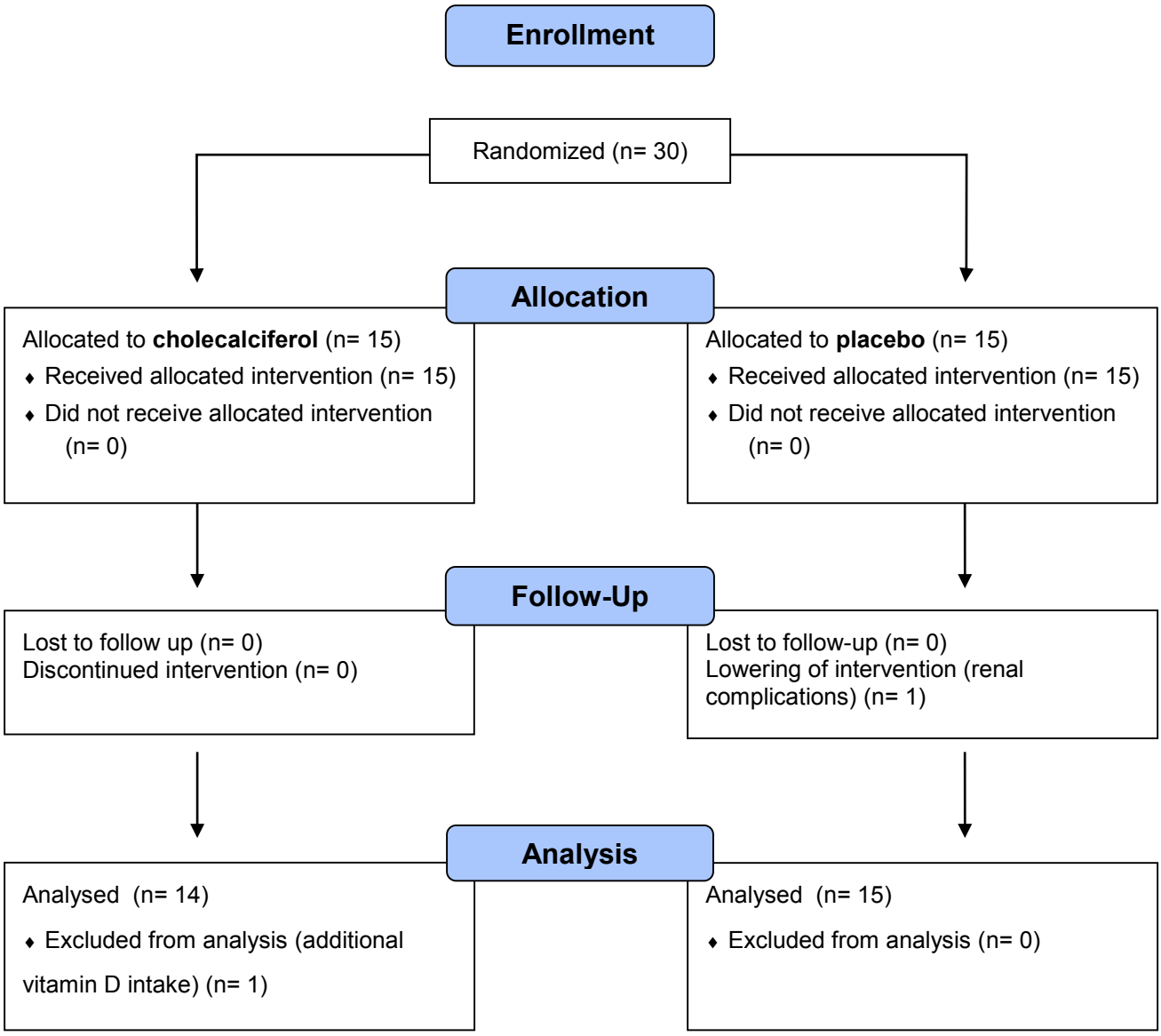

Figure 8: Adapted Consort Flow Diagram for the ADPP002 study
This diagram summarizes the progress through the different phases of the randomized, double blinded, placebo controlled trial. This includes enrollment, intervention allocation, follow-up phase and data analysis. In this RCT newly diagnosed children and adult patients with type 1 diabetes were included and it was registered at ClinicalTrials.gov (NCT01390480).

Adapted CONSORT 2010 Flow Diagram

Study ID: **ADPP003**

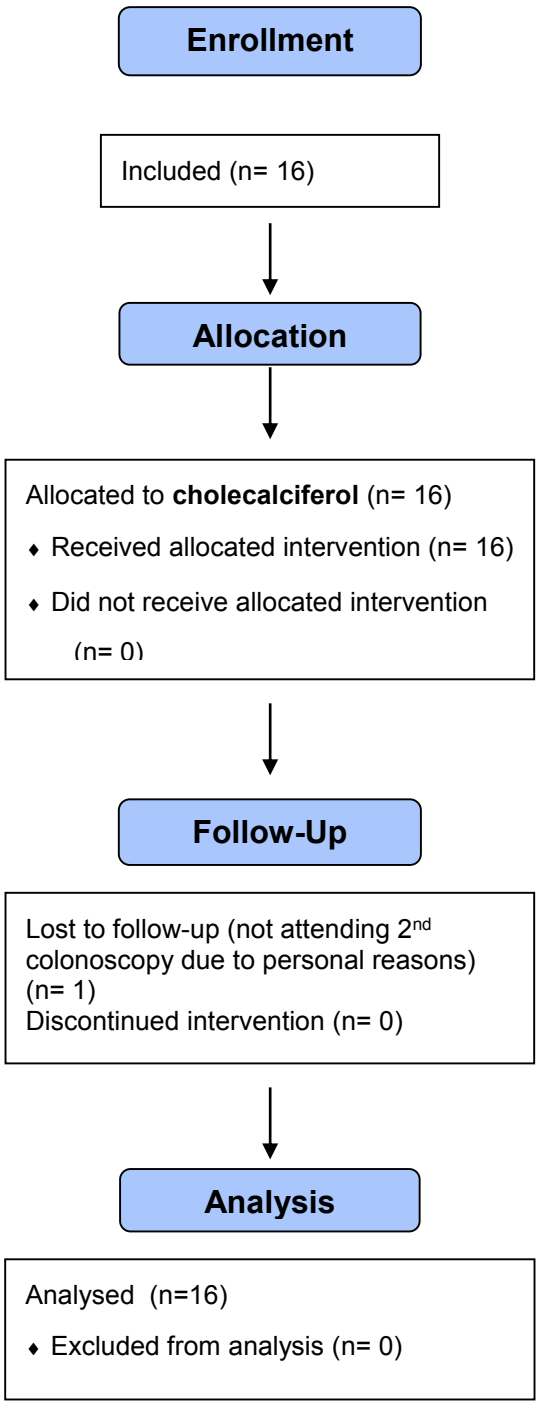

Figure 9: Adapted Consort Flow Diagram for the ADPP003 study
This diagram summarizes the progress through the different phases of the open pilot study. This includes enrollment, allocation, follow-up phase and data analysis. In this trial adult healthy participants were included and it was registered at ClinicalTrials.gov (NCT01538485).

Adapted CONSORT 2010 Flow Diagram

Study ID: **ADPP004**

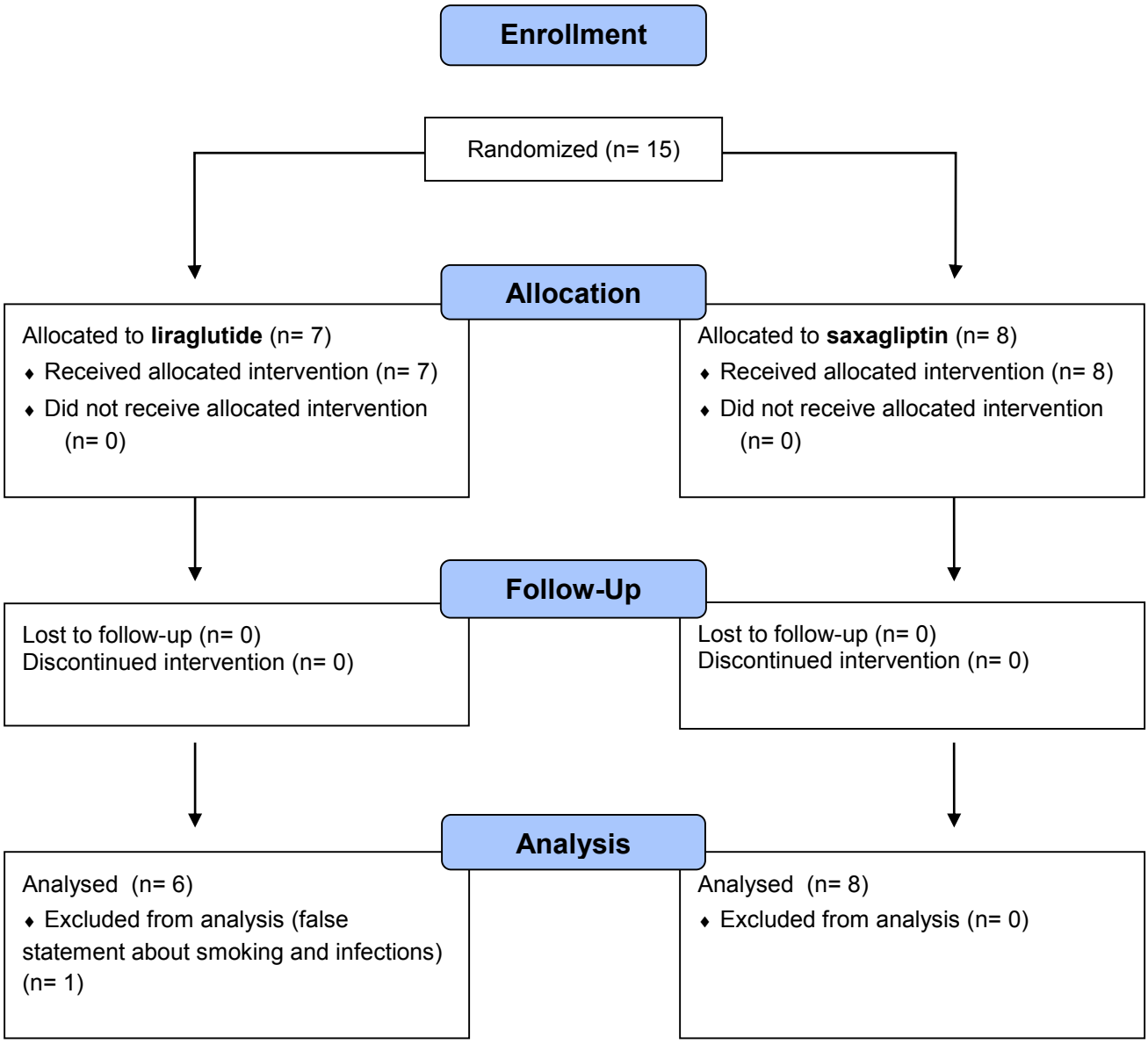

Figure 10: Adapted Consort Flow Diagram for the ADPP004 study
This diagram summarizes the progress through the different phases of the randomized, blinded pilot study. This includes enrollment, allocation, follow-up phase and data analysis. In this trial adult healthy participants were included and it was registered at ClinicalTrials.gov (NCT01782261).

Immunological assessment

Blood cell preparation for the assessment of immune parameters

Fresh heparinized whole blood was either directly stained for flow cytometric analysis or used for the isolation of peripheral blood mononuclear cells (PBMCs). Absolute numbers of subtypes of blood leukocytes were determined by using a hematological cell counter (Beckman Coulter, The Netherlands). For PBMC isolation, a density gradient cell separation medium (Histopaque Hybri Max 1077, Sigma-Aldrich, Germany) was used and cells were prepared according to the manufacturer's protocol. Isolated cells were washed twice with Hank´s balanced salt solution (HBSS, Life Technologies, Germany). PBMCs were either re-suspended in PBS (Thermo Fischer Scientific, USA) and used for FACS staining or re-suspended in X-vivo media completed with 10% human AB serum (Lonza, USA) for the performance of functional cell culture assays.

During establishment and optimization of PBMC isolation the viability of the PBMCs was tested using trypan blue staining (Sigma Aldrich, Germany) for microscopy and 7 AAD and Annexin V for FACS analysis of dead and/ or apoptotic cells. As viability of isolated cells was always above 98% at least, we left out this step for the following studies.

Tissue cell preparation for the assessment of immunological parameters

For mucus removal drawn biopsies from the intestinal tract were immediately transferred to a dithiothreitol/EDTA solution (Sigma, Germany and Life Technologies, Germany) and incubated for 15 min at 37°C under rotation. Then the tissue was finely sliced mechanically and digested for 1h (37°C) in a collagenase A solution (Roche, Germany). This was followed by passing the cell suspension through a 70-100 µm cell strainer (BD Biosciences, USA) in order to get single cell suspensions. Cells were collected in a tube containing cRPMI-10 media (Life Technologies, Germany). Single cells were pelleted by centrifugation and re-suspended in PBS (Thermo Fischer Scientific, Germany). Cell viability was monitored by staining an aliquot with 0.4% trypan blue solution (Sigma Aldrich, Germany) for microscopy. Cell suspensions obtaining viability above 90% were used for FACS staining of lamina propria mononuclear cells.

Multicolor cell staining and quantitative FACS analysis

Table 3 gives an overview about the FACS panels used in the different study setups. Staining of human PBMCs and analysis for $CD4^{pos}CD25^{high}FoxP3^{pos}CD127^{dim}$ regulatory T cells using the FACSCanto II flow cytometer was performed with the following antibodies: anti-CD4 FITC, anti-CD25 PE-Cy7 and anti-CD127 PE. After surface marker staining cells were treated with the human FoxP3 Buffer Set (BD Biosciences, USA) and intracellular markers were stained using anti FoxP3-AF647 or anti FoxP3-V450 and anti-Helios-AF647 (eBiosciences, USA). T cell subpopulations in whole blood were quantified by using the following monoclonal fluorochrome-conjugated antibodies: anti-CD3 V450, anti-CD4 PE, anti-CD8 PerCP-Cy5.5, anti-CD25 FITC, anti-CD45RO APC, anti-CD45RA PE-Cy7 and anti-CD45 APC-H7. Natural killer cells (NK), natural killer T cells (NKT), invariant natural killer T cells and B cells were quantified by using anti-CD3 V450, anti-CD45 APC-H7, anti-CD56 PE, anti-Vα24Jα18TCR (eBiosciences, USA) and anti-CD19 PE-Cy7 antibodies. Finally, plasmacytoid and myeloid dendritic cells were quantified by using anti-CD123 PE, anti-CD11c APC, anti-HLA-DR PE-Cy7, anti-CD45 APC-H7 antibodies and a ready-to-use lineage marker kit for the exclusion of all other blood cells.

Isolated cells from human gut biopsies were stained with the following fluorochrome-conjugated monoclonal antibodies: anti-CD3 PerCP-Cy5.5, anti-CD4 PE, anti-CD8 V500, anti-CD25 PE-Cy7 and anti-CD 127 FITC. For intracellular staining, cells were fixed and permeabilized using the human FoxP3 Buffer set and the anti-FoxP3 V450 (BD Biosciences, USA) and anti-Helios AF647 antibodies (eBiosciences, USA).

For the GLP1-R quantification 100µl of fresh whole blood was stained using the following monoclonal antibodies: anti-CD3 V450, anti-CD4 FITC, anti-CD45 APC-H7, anti- CD25 PE- Cy7, anti- CD19 PE- Cy7, anti- CD8 PerCp- Cy5.5, anti- CD14 PerCp- Cy5.5, anti- CD15 FITC, anti- CD45RA PE- Cy7 and anti- CD45RO APC. Intracellular staining of the transcription factor FoxP3 and the intracellular domain of the GLP1-R was done by using anti-FoxP3 V450 and anti- GLP-1R PE (R&D Systems, USA) monoclonal antibodies and the human FoxP3 buffer set for fixation and permeabilization (BD Biosciences, USA).

All monoclonal fluorochrome-conjugated antibodies in this section were purchased from BD-Biosciences (USA) if not stated otherwise. For the proper setting of gates and quadrants, isotype controls and the fluorochrome minus one (FMO) method (74) were used when needed. The number of events recorded per FACS analysis

depended on the subpopulation to be analyzed. For peripheral Tregs and $CD4^{pos}$T-cell subtype quantification at least 20 000 $CD4^{pos}$ cells were recorded. The analysis of other peripheral T-cell subtypes, B cells, monocytes, granulocytes and natural killer cells (NK) as well as natural killer T cells (NKT) was based on recording at least 300 000 events. For the assessment of subtypes of peripheral dendritic cells at least 350 000 events were recorded. FACS quantification of lamina propria associated immune cells was done by recording as much events as possible.

To avoid possible run to run laser performance variations, the used FACS system (FACS Canto II with FACS Diva software version 6.1.3) was controlled by day-to-day cytometer performance checks with BD Cytometer Setup and Tracking Beads (CST beads, BD Biosciences, USA). This enabled reproducible application settings. Furthermore, as the FACS was used for routine medical measurements, Rainbow Calibration Particles (8 peaks, BD Biosciences, USA) were used for routine calibration of the cytometer. Especially for Treg quantification, the intra-assay coefficients of variance (CV) was measured with PBMC samples from 5 different healthy volunteers, which was 4.5 %.

Cell sorting and suppression co-cultures

Suppressive capacity of regulatory T cells (Tregs) was assessed in a co-culture with autologous effector T cells (Teffs) and Tregs. $CD4^{pos}CD25^{high}CD127^{low/neg}$ Tregs and $CD4^{pos}CD25^{low/neg}CD127^{pos}$ Teffs were isolated using a FACSAria II cell sorter (BD Biosciences, USA). Viability of sorted cells was verified by 7-AAD/Annexin V staining and FACS quantification of life, dead or apoptotic cells. Suppressive co-cultures were only setup with cells containing at least 95% live cells. Within 1h after sorting Teffs were seeded in a 96-well U-bottom plate ($2.5x10^4$/well) and cultivated in the presence or absence of autologous Tregs at a 1:1 ratio and the presence of irradiated autologous PBMCs ($2.5x10^4$/well). Effector cells (Teffs) were stimulated by adding anti-CD3/CD28 coated microbeads at a concentration 1 bead/ cell (Invitrogen, USA) and incubated at 37°C and 5% CO_2 for 96 h. For the final 18h of culture Teff proliferation was monitored by analyzing ^{3}H-thymidine uptake (3H-thymidin from Biotrend, Germany), measured on a MicroBeta Trilux Counter (Perkin Elmer, USA).

For the assessment of the effects of *in-vitro* liraglutide treatment on the function of Tregs liraglutide ([10 µg/ml], Victoza®, Novo Nordisk, Denmark, diluted in sterile PBS) was added to the co-cultures prior to effector T cell stimulation.

Apoptosis measurement of Tregs and Teffs

Within one hour after sorting, $8x10^3$ Tregs or Teffs were stained with 7AAD and Annexin-V APC (BD Biosciences, USA) according to manufacturer's instructions and analyzed on a FACSCanto II flow cytometer for apoptosis quantification.

Th1, Th2, Th17 quantification

Isolated and washed PBMCs were seeded in culture media (cRPMI) and stimulated with anti-CD3/CD28 beads (1×10^5 cells/well) for 24h. Golgi Stop (BD Biosciences, USA) was added during the last 4h according to manufacturer's instructions. For FACS quantification, cells were harvested and stained with anti-CD4 V450 (eBiosciences, USA), anti-CD2 PE-Cy7 (BD Biosciences, USA) and washed with PBS containing 10% heat inactivated FBS (Sigma Aldrich, Germany). Intracellular staining was performed using the human FoxP3 buffer set from BD Biosciences (USA) and monoclonal antibodies specific for anti-IL17 PE (eBiosciences, USA), anti IFN-γ FITC (BD Biosciences, USA) and anti-IL4 APC (eBiosciences, USA).

HEK-293 cell culture

The specificity of the used anti-GLP-1R antibody was tested by staining human embryonic kidney cells (HEK-293) as a negative control for GLP-1R. HEK-293 cells were purchased from the center of medical research, Graz (ZMF) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Germany) containing 10% heat inactivated fetal bovine serum (FBS, Life Technologies, USA), 2mM L-Glutamine and Penicillin/Streptomycin (Life Technologies, USA). Cell aliquots were frozen at -80°C in FBS (Life Technologies, USA) containing 10% dimethyl sulfoxide (Sigma-Aldrich, Germany) at passage 23 and all experiments were carried out with thawed cells of this passage. Prior to antibody staining, dead cells were labelled with fixable viability dye eFluor 506 (eBiosciences, USA) according to the manufacturer´s instructions. For GLP1-R staining, the same procedure as mentioned above was used.

Quantification of GLP1-R mRNA

Purchased pure RNA from human $CD19^{pos}$ B cells, $CD4^{pos}CD25^{pos}$ T cells, and monocytes (Miltenyi Biotech, Germany) was used for cDNA synthesis using a cDNA

synthesis kit (Qiagen, UK) including a negative control. GLP1-R expression analysis was quantified using the reference gene β-actin with a TaqMan 5' exonuclease assay, including the forward 5-TCAAGGTCAACGGCTTATTAG-3 and reverse 5-TAACGTGTCCCTAGATGAACC-3 primers (Applied Biosystems, USA) on a real time PCR cycler (LC480, Roche, Germany). These experiments were instructed and guided by Dr. Natascha Schweighofer from the division of Endocrinology and Diabetology.

In vitro cell cultures with liraglutide

For the assessment of the effect of GLP-1 treatment on human immune cells, liraglutide (Victoza®, Novo Nordisk, Denmark) or the matrix containing 1.42 mg disodium phosphate dehydrate, 14 mg propylene glycol, 5.5 mg phenol and aqua bidest (all purchased from Sigma Aldrich, Germany), was added to PBMC cultures from healthy participants. All measurements were carried out in 3 independent experiments with cells from different healthy donors. Liraglutide or matrix were used in the following concentrations: 0µg, 1µg, 5µg, 10µg, 20µg, 40µg and 80 µg/ml. Cells were left unstimulated or were stimulated by the addition of the human anti CD3/CD28 dynabeads (1 bead/ cell, Invitrogen, USA) and were cultivated in cRPMI 1640 media containing 10% heat inactivated FBS, 2mM L- Glutamine and Penicillin/Streptomycin (all from Life Technologies, USA) for 96 h at 37 °C and 5 %CO_2. Finally, cell proliferation was quantified by the use of the Vialight Plus Cell Proliferation Assay (Lonza, Belgium). The assay was performed according to the manufacturer´s instructions. Bioluminescent measurement of ATP was carried at a LUMIstar Optima Microplate Luminometer (Tecan, Austria).

Additionally, freshly isolated PBMCs were treated with liraglutide ([10 µg/ml], Victoza®, Novo Nordisk, Denmark, diluted in sterile PBS) or matrix for 96h and apoptosis and cytokine production of the cultivated cells was analyzed as described above.

Clinical assessment

Recruitment and support of participants

The whole processes of recruitment, clinical assessment and clinical support of the study participants were done by the following persons: Dr. Gerlies Treiber (Div. of Endocrinology and Diabetology, Graz), Dr. Martin Tauschmann (Div. of Endocrinology and Diabetology, Graz), Dr. Elke Fröhlich-Reiterer (Department of Pediatrics, Graz), Dr. Anja Ribitsch (Div. of Endocrinology and Diabetology, Graz), Dr. Maria Fritsch (Department of Pediatrics, Vienna), Dr. Birgit Rami-Merhar (Department of Pediatrics, Vienna), Dr. Claudia Steigleder-Schweiger (Department of Pediatrics, Salzburg), Christine Neuper (Div. of Endocrinology and Diabetology, Graz) and Silvia Leitgeb (Div. of Endocrinology and Diabetology, Graz).

Measurements of clinical safety parameters

In all studies the following safety parameters were assessed by standardized routine analysis: complete blood cell count, C-reactive protein (CRP) levels, blood electrolyte levels, serum calcium levels, serum urea levels, serum uric acid levels, serum proteins (whole proteins and albumin), serum liver parameters, serum cholesterin, serum triglycerides, urine calcium, urine calcium/creatinine ratio, urine potassium, urine natrium and urine albumin. Additionally, serum C-peptide levels, serum HbA1c levels, serum insulin levels, serum glucose levels and different diabetes-related auto-antibodies (against GADA, IA2, ZnT8) were measured in all T1D patients and in healthy participants serving as controls for the patients. In a subgroup of participants, older than 14 years, PTH levels were measured regularly during the whole study-duration by standard laboratory methods. Serum 25(OH)D (calcifediol) was determined with a commercially available ELISA (IDS, UK) for all studies where cholecalciferol supplementation was administered (ADPP002, ADPP003, healthy control study). Additionally, $1,25(OH)_2D_3$ was quantified by the use of a combination of immunopurification and an automated chemiluminescent immunoassay (IDS, Germany) in the serum of individuals participating in the healthy vitamin D study.

Metabolic outcome measurements

For studies assessing the difference between healthy controls and T1D patients (healthy control study, ADPP001) or measuring the effects of a cholecalciferol

treatment (ADPP002) a mixed meal tolerance test (MMTT) was performed. Fortimel Complete® (Nutricia, Austria) was taken orally after a 12h overnight fast in the amount of 6 ml/kg bodyweight (maximum of 360 ml). Blood was drawn at 5 consecutive time points (0, 30, 60, 90 and 120 min) to analyze fasting and stimulated serum C-peptide levels.

Statistical methods

Prior to data analysis, all metric outcome variables were checked for normality by means of a Shapiro–Wilk test. Data that were not normally distributed were log transformed for statistical analysis. Data from healthy participants receiving cholecalciferol or placebo treatment for 12 weeks were analyzed with a general linear model (GLM) with repeated measurements. To compare vitD and placebo groups, data were compared using a Welch's t test (unequal variances), a Student's t test, a Mann–Whitney U test for unpaired data or a Wilcoxon signed-rank test for paired data. Pearson correlation was used to analyze supplementation and effect data. All statistical analysis was performed using SPSS version 19.0 software (SPSS Inc., USA) and p-values <0.05 were considered statistically significant and when necessary significance levels were adjusted using Bonferroni corrections.

Data from T1D patients were analyzed using the two-way repeated-measures analysis of variance to determine changes between the cholecalciferol and placebo groups over 12 month of treatment. For group comparisons the Student's t-test was applied for normally distributed data and the non-parametric Mann-Whitney U-test was used if data deviated from normality. The level of significance was set to 5% for all tests. Statistical analyses were performed using R 2.13.

Study ID	Panel ID	Fluorochrome								
		FITC	**PE**	**APC**	**PE-Cy7**	**PerCP-Cy5.5**	**AF-647**	**APC-H7**	**V450**	**V500**
Healthy VitD	Treg	CD4	CD127		CD25		FoxP3			
	T cells	CD25	CD4	CD45RO	CD45RA	CD8		CD45	CD3	
	IPT	CD15	CD56	CD34	CD19	CD14		CD45	CD3	
	DC	Lin1	CD123	CD11c	HLA-DR			CD45		
	Sorting	CD4	CD127		CD25					
	Apoptosis			AnnexinV		7AAD				
ADPP001	Treg	CD4	CD127		CD25		FoxP3/ Helios			
	T cells	CD25	CD4	CD45RO	CD45RA	CD8		CD45	CD3	
	IPT		CD56	CD34	CD19			CD45	CD3	
	DC	Lin1	CD123	CD11c	HLA-DR			CD45		
	Sorting	CD4	CD127		CD25					
	Apoptosis			AnnexinV		7AAD				
	GLP1R	CD4	GLP1R		CD25	CD8			FoxP3	
		CD4	GLP1R	CD45RO	CD45RA	CD8		CD45	CD3	
		CD15	GLP1R		CD19	CD14		CD45	CD3	
	pSTAT 5	CD4	CD127		CD25		pSTAT5		FoxP3	
ADPP002	Treg	CD4	CD127		CD25		FoxP3/ Helios		FoxP3	
	T cells	CD25	CD4	CD45RO	CD45RA	CD8		CD45	CD3	
	IPT		CD56		CD19			CD45	CD3	
	DC	Lin1	CD123	CD11c	HLA-DR			CD45		
	Sorting	CD4	CD127		CD25					
	Th cells	IFN-g	IL-17	IL-4	CD2				CD4	
	Apoptosis			AnnexinV		7AAD				

Study ID	Panel ID	Fluorochrome								
		FITC	**PE**	**APC**	**PE-Cy7**	**PerCP-Cy5.5**	**AF-647**	**APC-H7**	**V450**	**V500**
ADPP003 blood	Treg	CD4	CD127		CD25		Helios		FoxP3	
	T cells	CD25	CD4	CD45RO	CD45RA	CD8		CD45	CD3	
	IPT		CD56	iNKT				CD45	CD3	
	DC	Lin1	CD123	CD11c	HLA-DR			CD45		
	Th cells	IFN-g	IL-17	IL-4	CD2				CD4	
ADPP003 biopsies	Treg	CD127	CD4		CD25	CD3	Helios		FoxP3	CD8
ADPP004	Treg	CD4	CD127		CD25		FoxP3			
	T cells	CD25	CD4	CD45RO	CD45RA	CD8		CD45	CD3	
	NK,NKT		CD56	iNKT				CD45	CD3	
	DC	Lin1	CD123	CD11c	HLA-DR			CD45		
	Th cells	IFN-g	IL-17	IL-4	CD2				CD4	

Table 3: Overview for the monoclonal antibodies and fluorochomes used in the clinical studies included in this thesis. Study IDs are given as described in the text. Abbreviations for the fluorochromes: FITC: Fluorescein isothyocyanate, PE: Phycoerythrin, APC: Allophycocyanin, PE-Cy7: Phycoerythrin-cyanin dye 7, Per-CP-Cy5.5: Peridinin chlorophyll-cyanin dye 5.5, AF-647: Alexa Fluor 647, APC-H7: Allophycocyanin-H7, V450: violet dye 450, V500: violet dye 500.

Results

Study compliance of healthy volunteers and patients

In the cohort study (ID: ADPP001) samples from 373 participants were investigated in total. All volunteers had to perform one single visit, therefore the study compliance was no limiting factor.

For the interventional studies (IDs: Healthy VitD, ADPP002 - 004), the study compliance is given within the detailed description of results and summarized in adapted Consort flow diagrams (see Tab. 7-10). Demographics for all studies are given within the detailed description of results.

Differences in the percentage and function of peripheral immune cells between healthy controls, first degree relatives and T1D patients

Study ID: ADPP001

In total 373 participants were involved in this cross sectional study (Fig. 11). Within these participants 179 suffered from T1D (varying disease durations), 58 were first degree relatives (FDR) of the involved patients and 136 were non related healthy controls (Fig. 11).

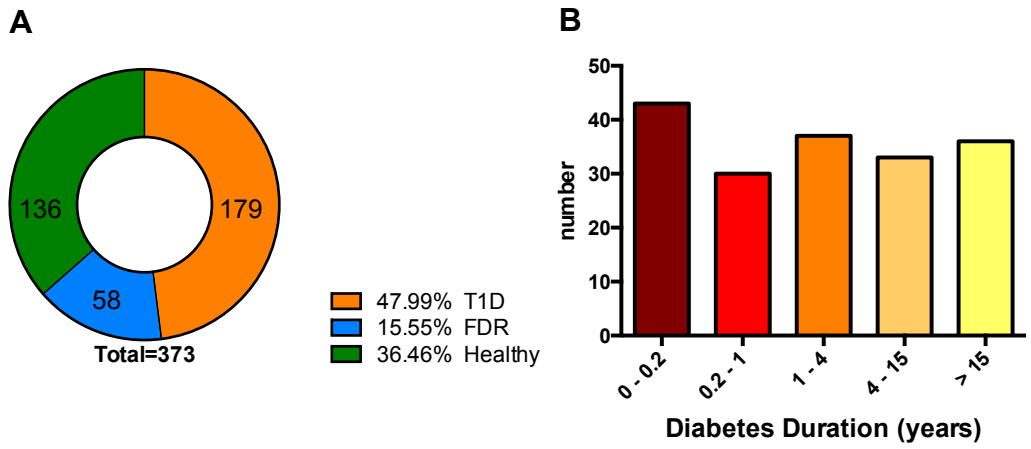

Figure 11: Study participants involved in the cross-sectional cohort study, called Austrian Diabetes Prevention Program 001 (ADPP001). **A.** Parts of the whole graph with data given as total numbers and percentage (figure legend) for the 3 defined groups; T1D: type 1 diabetes patients; FDR: first degree relatives; Healthy: healthy unrelated controls. **B.** Bar chart with total numbers of T1D-patients sorted by the category of disease duration in years.

Demographics for all participants and for subgroups defined according to age and diabetes duration are shown in tables 4-6. Not all laboratory measurements where done with the whole cohort as subgroups for special FACS quantifications were defined (e.g. GLP1-R staining). Demographics for special analysis are given within the subsequent results sections.

All participants	Healthy	FDR	T1D
n	136	58	179
Gender (% female)	52	62	42
BMI (kg/m^2)	23 (20-26)	22 (19-25)	22 (20-24)
Age (years)	27 (22-34)	24 (14-46)	25 (15-40)
Serum 25(OH)D (ng/ml)	26 (18-34)	33 (28-37)	30 (21-38)
T1D duration (years)	-	-	2.1 (0.3-12)
HbA1C (% Hb)	5.1 (4.9-5.3)	5.2 (5.0-5.4)	7.3 (6.6-8.3)
NLR	1.39 [1.03-1.82]	1.56 [1.28-1.99]	1.61[1.20-2.08]

Table 4: Demographics of all participants included in the ADPP001 cross-sectional cohort study. BMI: body mass index, 25(OH)D: 25-hydroxyvitamin D, HbA1c: glycated hemoglobin; NLR: neutrophil to lymphocyte ratio; normally distributed data are given in mean ± SD, not normally distributed data are given in median (+ interquartile range).

Recently diagnosed cohort	Healthy	FDR	T1D
n	86	37	110
Gender (% female)	50	59	45
BMI (kg/m^2)	22 (20-25)	20 (18-23)	21 (17-24)
Age (years)	23 (20-27)	17 (12-23)	18 (12-34)
Serum 25(OH)D (ng/ml)	25 (18-33)	32.5 (29-35)	30 (20-38)
T1D duration (years)	-	-	0.5 (0.2-2)
HbA1C (% Hb)	5.1 (4.9-5.3)	5.1 (29-35)	7.4 (6.4-8.4)
NLR	1.28 [0.93-1.66]	1.52 [1.21-2.16]	1.34 [1.06-1.67]

Table 5: Demographics of a subgroup analysis, including just recently diagnosed patients and age- and gender-matched healthy participants from the ADPP001 cross-sectional cohort study. BMI: body mass index, 25(OH)D: 25-hydroxyvitamin D, HbA1c: glycated hemoglobin; NLR: neutrophil to lymphocyte ratio; normally distributed data are given in mean ± SD, not normally distributed data are given in median (+ interquartile range).

Newly diagnosed cohort	Healthy	FDR	T1D
n	30	27	43
Gender (% female)	51	54	42
BMI (kg/m^2)	21 (16-23)	19 (18-22)	19 (16-22)
Age (years)	17 (11-20)	14 (11-19)	15 (11-26)
Serum 25(OH)D (ng/ml)	30 (18-36)	33 (27-35)	28 (19-33)
T1D duration (years)	-	-	0.2 (0.1-0.2)
HbA1C (% Hb)	5.1 (4.8-5.3)	5.0 (4.9-5.4)	7.7 (6.9-8.4)
NLR	1.40 [1.01-1.69]	1.57 [1.32-1.95]	1.82 [1.29-2.36]

Table 6: Demographics of a subgroup analysis, including just newly diagnosed patients and age- and gender-matched healthy participants from the ADPP001 cross-sectional cohort study. BMI: body mass index, 25(OH)D: 25-hydroxyvitamin D, HbA1c: glycated hemoglobin; NLR: neutrophil to lymphocyte ratio; normally distributed data are given in mean ± SD, not normally distributed data are given in median (+ interquartile range).

Significantly increased percentage of CD19^{pos} B cells in T1D patients compared to healthy controls

Comparing all data from unrelated heathy volunteers and T1D patients showed significant differences between in the % of CD19^{pos} B cells within the lymphocytes in the peripheral blood. The FACS gating strategy is shown in figure 12. The % of CD19^{pos} B cells was significantly increased in lymphocytes from T1D patients (median + IQR: 13.45% (10.53-16.72)) when compared to healthy unrelated controls (11.27 (8.38-14.89)). Data from first degree relatives of the investigated patients showed no significant difference from control data (Fig. 12 D).

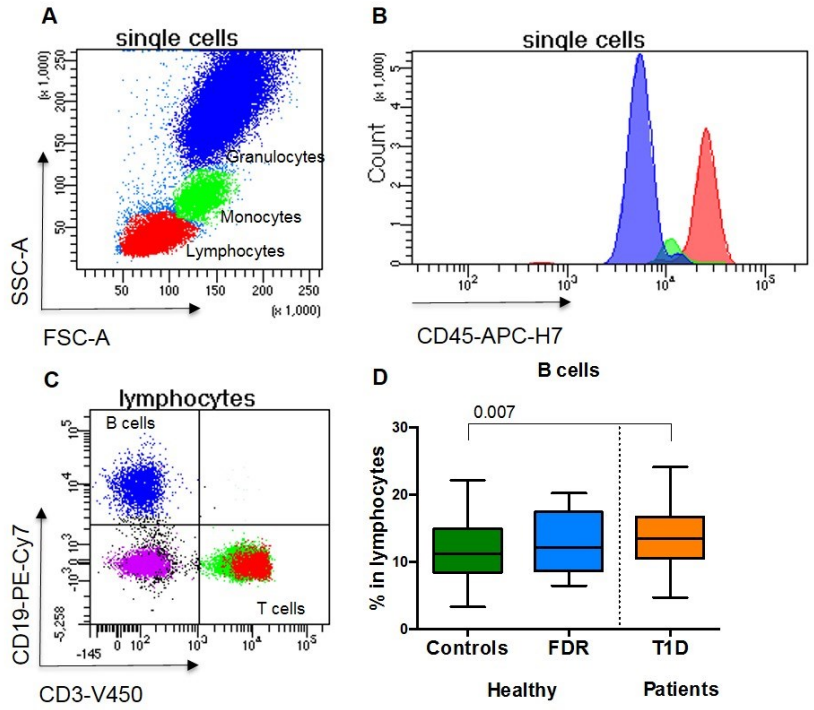

Figure 12: Peripheral CD19^{pos} B cells **A.** FACS dot plot showing lymphocytes (red), monocytes (green) and granulocytes (blue) in a sample of whole blood according to their different sizes and granularity. **B.** FACS histogram showing the positive signals for the CD45 marker in lymphocytes (red), monocytes (green) and granulocytes (blue). **C.** Gating of CD19^{pos} B cells (blue) and CD3 positive T cells (green and red) within the lymphocyte population. **D.** Box and whisker plots showing the data for unrelated controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values, the p-value is shown on top of the horizontal line.

<u>No differences in the percentage of the big subtypes of T cells within lymphocytes between healthy controls and T1D patients</u>

CD4^{pos} helper T cells

Analyzing the whole data set revealed no differences in the percentage of lymphocytes expressing the CD4 molecule when comparing healthy participants (45.8 ± 7.8 %), first degree relatives (44.8 ± 7.9 %) and T1D patients (45.0 ± 8.1%) (Fig. 13 C). Subgroup analysis according to disease duration of the patients also revealed no differences between the three groups (data not shown).

CD8^{pos} cytotoxic T cells

Analyzing the whole data set revealed no differences in the percentage of lymphocytes expressing the CD8 molecule between healthy participants (24.5 ± 6.7

%), first degree relatives (24.5 ± 6.4 %) and T1D patients (24.9 ± 6.3%, Fig. 13 D). Subgroup analysis according to age and diabetes duration of the patients also revealed no differences between the three groups (data not shown).

CD4CD8 double positive T cells

Analyzing the whole data set revealed no differences in the percentage of CD4CD8 double positive T cells within lymphocytes when comparing data from unrelated healthy controls (0.45 ± 0.32%), first degree relatives (0.75 ± 0.88%) and T1D patients (0.69 ± 1.48%, Fig. 13 E).

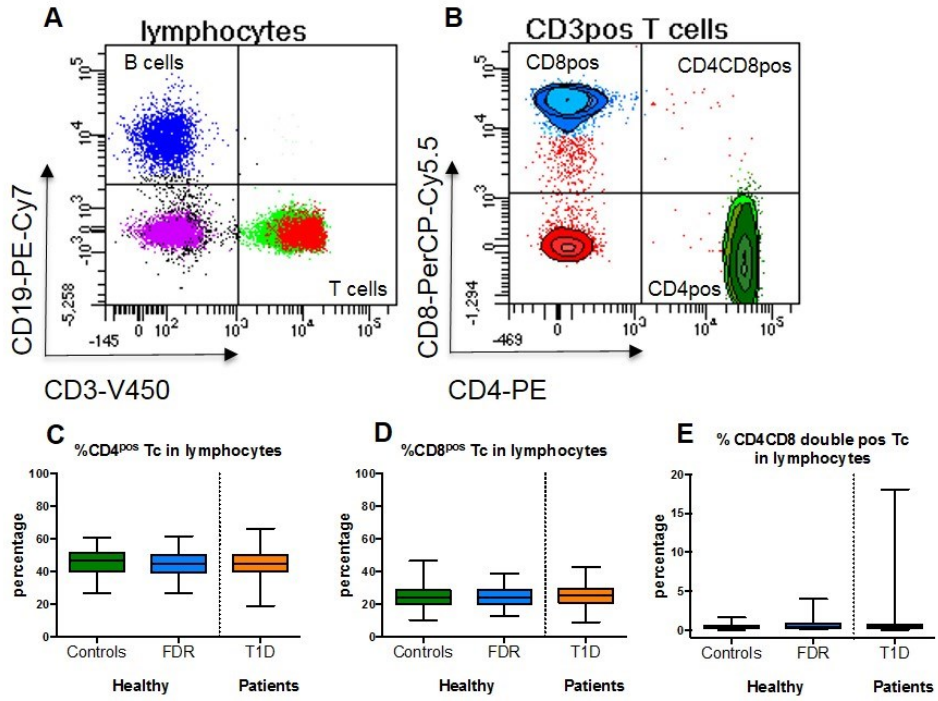

Figure 13: CD4^{pos} T helper cells, CD8^{pos} cytotoxic T cells and CD4CD8 double positive T cells **A.** Gating of CD19 positive B cells (blue) and CD3 positive T cells (green and red) within the lymphocyte population. **B.** Gating of CD4 positive T cells (green), CD8 positive T cells (blue) and CD4/CD8 double positive T cells within all CD3 positive lymphocytes. **C.** Box and whisker plots showing the data for CD4^{pos} T cells **D.** Box and whisker plots showing the data for CD8^{pos} T cells and **E.** Box and whisker plots showing the data for CD4CD8 double positive T cells from unrelated controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values.

Differences in naive and memory subtypes of CD4^{pos} T cells between T1D patients and healthy volunteers are correlated to age

Comparing all data from heathy volunteers and T1D patients showed highly significant differences in the % of naive CD45RA^{pos} T cells within the CD4^{pos} population. The % of naive CD45RA^{pos} cells within CD4^{pos}T helper cells was significantly decreased in T1D patients (45.0 ± 16%) when compared to healthy controls (52.9 ± 11.1%, p<0.001; Fig. 14 C). In contrast, memory CD45RA^{neg}CD45RO^{pos} expressing cells within the T helper cell population were significantly increased in T1D patients (47.25 ± 16.32%) when compared to healthy controls (42.23 ± 11.77%, p=0.009; Fig. 14 D). However, further analysis showed a strong correlation between age and naive and memory phenotype of CD4^{pos} T cells in all three investigated groups (Fig. 15, shown for healthy participants and T1D patients).

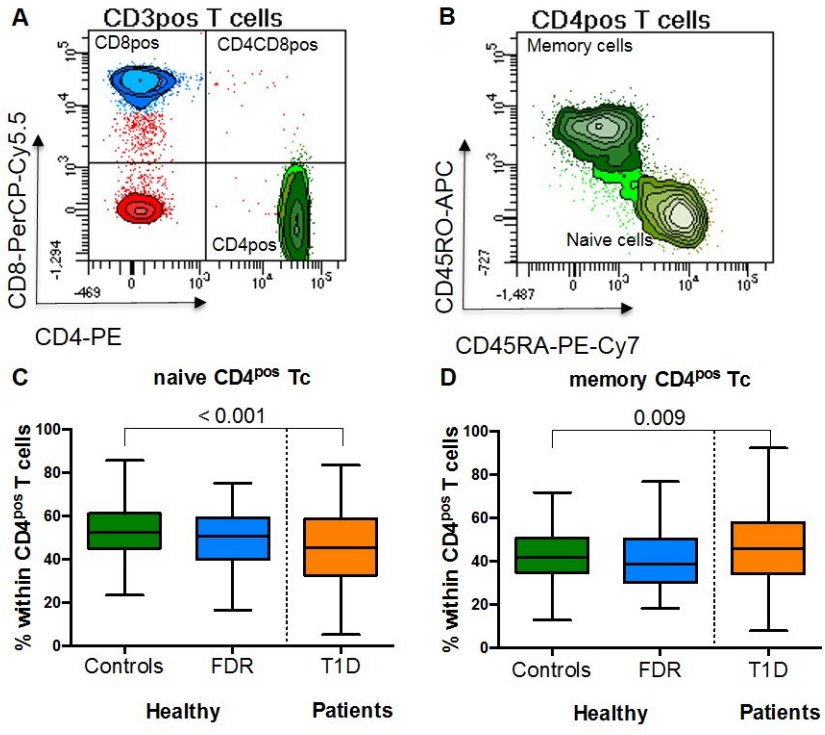

Figure 14: Naive and memory CD4^{pos} T helper cells. **A.** Representative FACS plot for the gating of CD4^{pos} T cells (green), CD8 positive T cells (blue) and CD4/CD8 double positive T cells within all CD3^{pos} lymphocytes **B.** Representative FACS plot for the quantification of naive cells (CD45RA^{pos}CD45RO^{neg}) and memory cells (CD45RA^{neg}CD45RO^{pos}) within all CD4^{pos} T cells. **C.+D.** Box and whisker plots showing the data for naive (C) and memory (D) CD4^{pos} T cells from unrelated healthy controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values. p-values are shown on top of the horizontal lines.

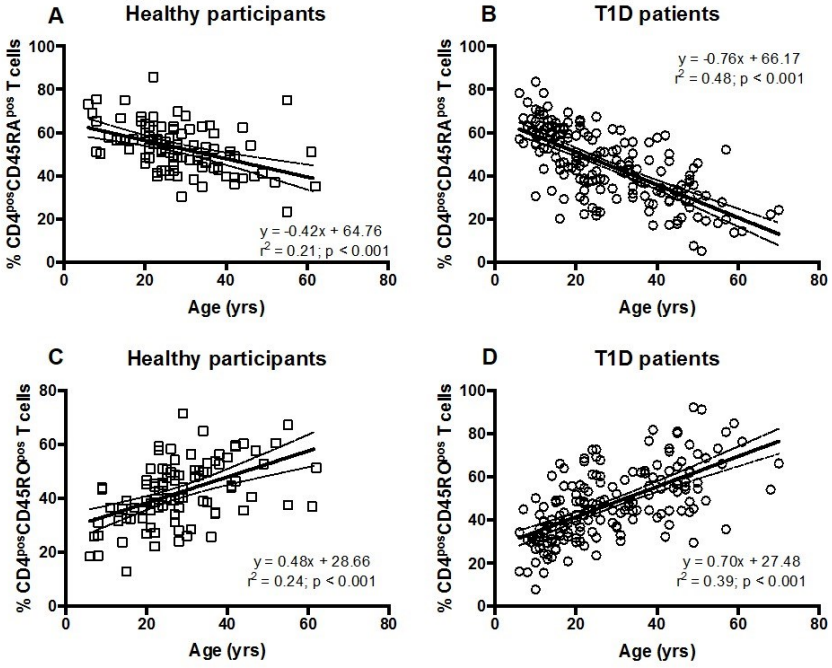

Figure 15: The % naive and memory $CD4^{pos}$ T cells are correlated to age in healthy controls and T1D patients **A. + B.** Pearson correlation of age and % naive $CD4^{pos}$ T cells for samples from healthy controls (A) and T1D patients (B) **C. + D.** Pearson correlation of age and % memory $CD4^{pos}$ T cells for samples from healthy controls (C) and T1D patients (D). Lines indicate regression and 95% CI, r^2 and p- values are given within the graphs.

No significant differences in the percentage of naive and memory $CD8^{pos}$ cytotoxic T cells

No significant differences were found in the percentage of naive $CD45RA^{pos}$ and memory $CD45RO^{pos}$ within $CD8^{pos}$ cytotoxic T cells between healthy controls (naive T cells: 60.15 ± 12.69%; memory T cells: 32.25 ± 13.24%) and T1D patients (naive T cells: 57.85 ± 15.55%; memory T cells: 32.67 ± 15.45%; Fig. 16). The FACS gating strategy is shown in Fig. 16 A + B.

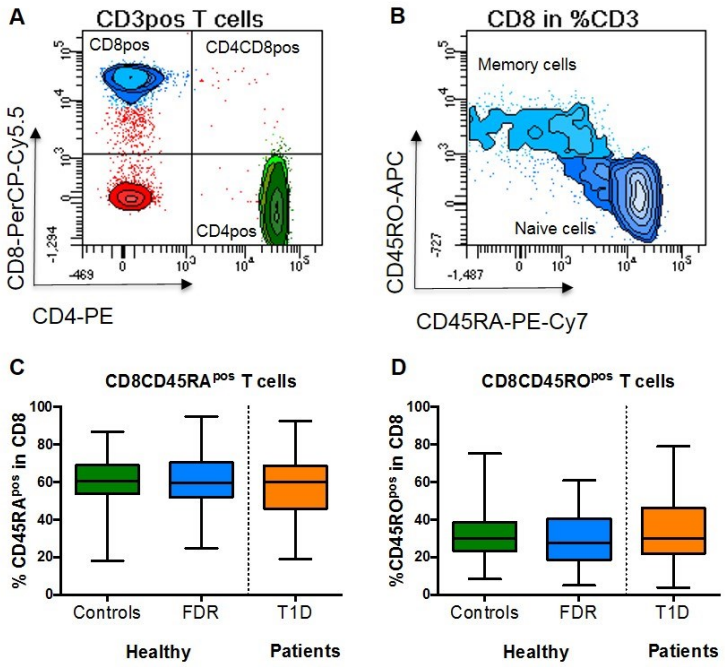

Figure 16: Representative FACS plots for the gating of naive and memory $CD8^{pos}$ cytotoxic T cells. **A.** FACS dot plot for the gating of $CD8^{pos}$ T cells (blue population) within peripheral $CD3^{pos}$ T cells **B.** representative FACS plot for the quantification of $CD45RO^{pos}$ memory cells (light blue) and $CD45RA^{pos}$ naive cells (dark blue) within $CD8^{pos}$ T cells **C.+ D.** Box and whisker plots showing the data for naive (C) and memory (D) $CD8^{pos}$ T cells from unrelated healthy controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values.

Subsequent data analysis revealed a strong correlation between age and expression of CD45RA and CD45RO protein on $CD8^{pos}$ cytotoxic T cells (Fig. 17).

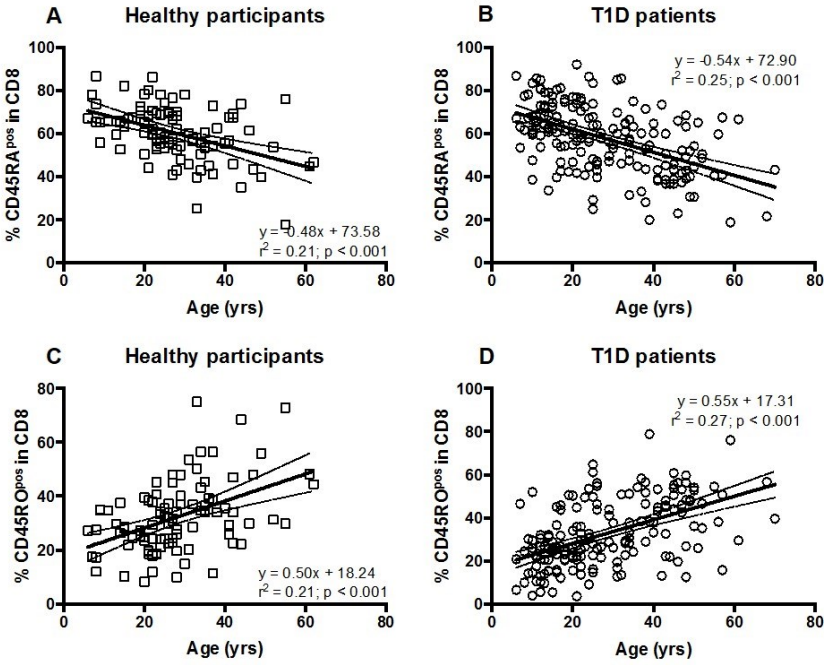

Figure 17: The % naive and memory $CD8^{pos}$ T cells are correlated to age in healthy controls and T1D patients. **A. + B.** Pearson correlation of age and % naive $CD8^{pos}$ T cells for samples from healthy controls (A) and T1D patients (B) **C. + D.** Pearson correlation of age and % memory $CD8^{pos}$ T cells for samples from healthy controls (C) and T1D patients (D). Lines indicate regression and 95% CI, r^2 and p values are given within the graphs.

<u>No differences in the % of peripheral Tregs between T1D patients and healthy volunteers</u>

The percentage of regulatory T cells, defined as $CD4^{pos}CD25^{hi}FoxP3^{pos}CD127^{neg/low}$ expressing T cells, was not different between T1D patients (median + IQR: 5.0 [3.8-5.9]) and healthy volunteers, neither when compared to healthy unrelated controls (4.9 [4.1-5.7]), nor when compared to first degree relatives of the patients (5.2 [4.2-5.7]). A representative FACS plot and the results for the % Treg for all investigated patients, healthy controls and FDR are shown in Fig. 18. Analyzing data from patients with different disease duration that were included in this cross-sectional study design revealed no changes in the % of Tregs within the increasing duration of disease (Fig. 18 C). Also no correlation of % Treg and serum-levels of C-peptide, HbA1C, 25(OH)D or fasting glucose was found (Fig. 19).

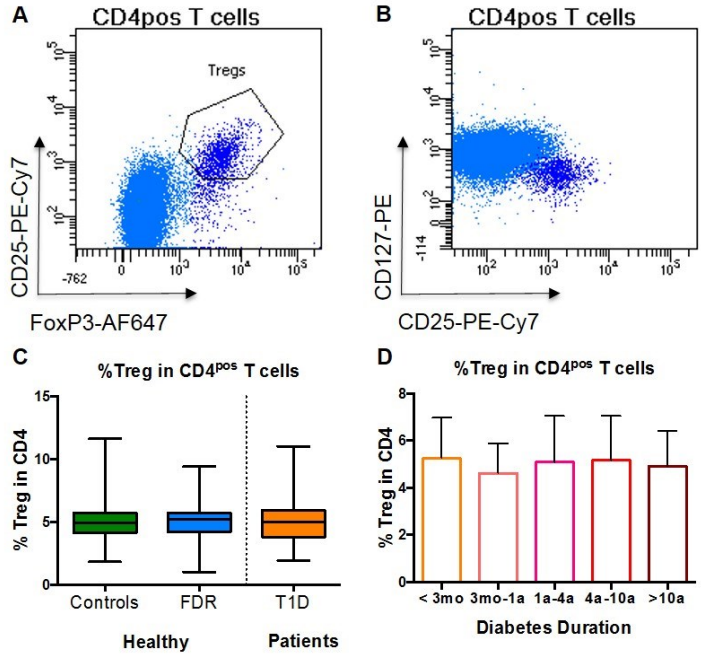

Figure 18: $CD4^{pos}$ regulatory T cells (Treg) in the peripheral blood. **A.** Representative FACS dot plot for the quantification of $CD4^{pos}CD25^{hi}CD127^{dim}FoxP3^{pos}$ Tregs within peripheral $CD4^{pos}$ T cells **B.** FACS dot plot for the proof of $CD127^{dim}$ expression of gated Tregs (dark blue) **C.** Box and whisker plots showing the data for % Treg within $CD4^{pos}$ T cells from unrelated healthy controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values **D**. Bar chart showing the mean % Tregs within $CD4^{pos}$ T cells from T1D patients with data sorted according to diabetes duration. Data given as mean + SD.

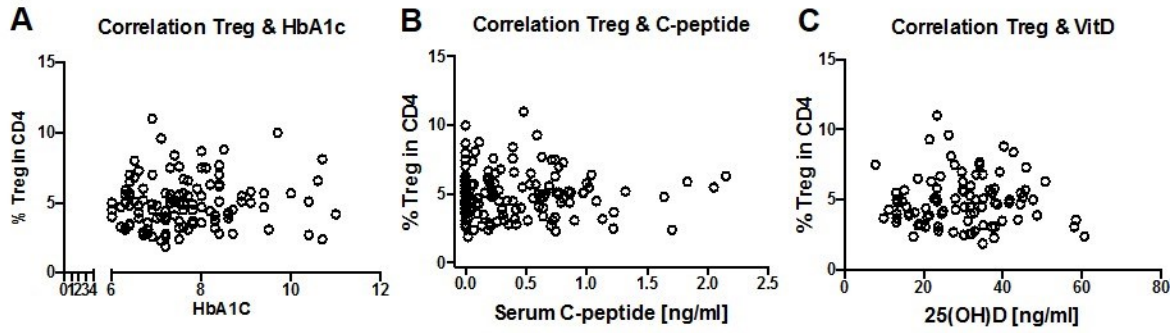

Figure 19: No correlation between the %Tregs within $CD4^{pos}$ T cells and HbA1c, vitamin D or C-peptide levels in the peripheral blood of T1D patients. **A**. Graph showing the Pearson correlation of the level of HbA1C and % Treg from T1D patients. **B.** Pearson correlation of the level of serum C-peptide and % Treg from T1D patients **C**. Pearson correlation of the level of serum vitamin D and % Treg from T1D patients.

Subgroup analysis based on differences in age and diabetes duration confirmed these results. For example, there were no significant differences in the % of peripheral Treg between healthy children and children with new onset T1D (defined by a diabetes duration below 3 months) or healthy adults and new onset T1D patients (data not shown).

Capacity of Tregs to suppress effector T cells is significantly decreased in T1D patients

In all investigated groups, isolated peripheral Tregs where able to reduce the proliferation of stimulated autologous CD4pos effector T cells significantly in an ex-vivo co-culture experiment (Fig. 20). However, the suppressive capacity of Tregs, defined as the % of reduction of Teff cell proliferation in the presence of autologous Tregs (see formula Fig. 20), was significantly decreased in the T1D group (32 ± 29%) when compared to healthy participants (40 ± 17%; p = 0.019; Fig. 21 A). Standard deviation was high in the T1D group, which was the only group containing Tregs that had no suppressive capacity at all or even increased proliferation of effector cells (Fig. 21 A). No correlation to age was found when data from healthy participants (Fig. 21 C) and T1D patients (Fig. 21 D) were analyzed. Furthermore in T1D patients no correlation between serum C-peptide level, 25(OH)D level, HbA1C level, diabetes duration and % suppression was found (Fig. 22).

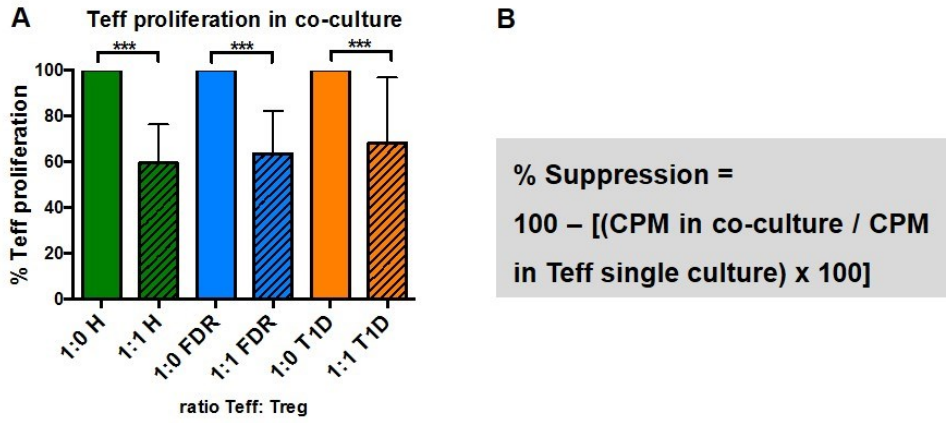

Figure 20: Suppression of effector cell proliferation by Tregs **A**. Bar graphs showing the % reduction of effector T cell proliferation caused by the addition of autologous Tregs in the co-culture. Data from healthy unrelated controls are shown in green, data from first degree relatives are shown in blue and data from T1D patients are given in yellow. Dashed bars indicate co-culture data. p-values are shown on top of the horizontal lines **B.** Equation for the calculation of % suppression. CPM = radioactive counts per million.

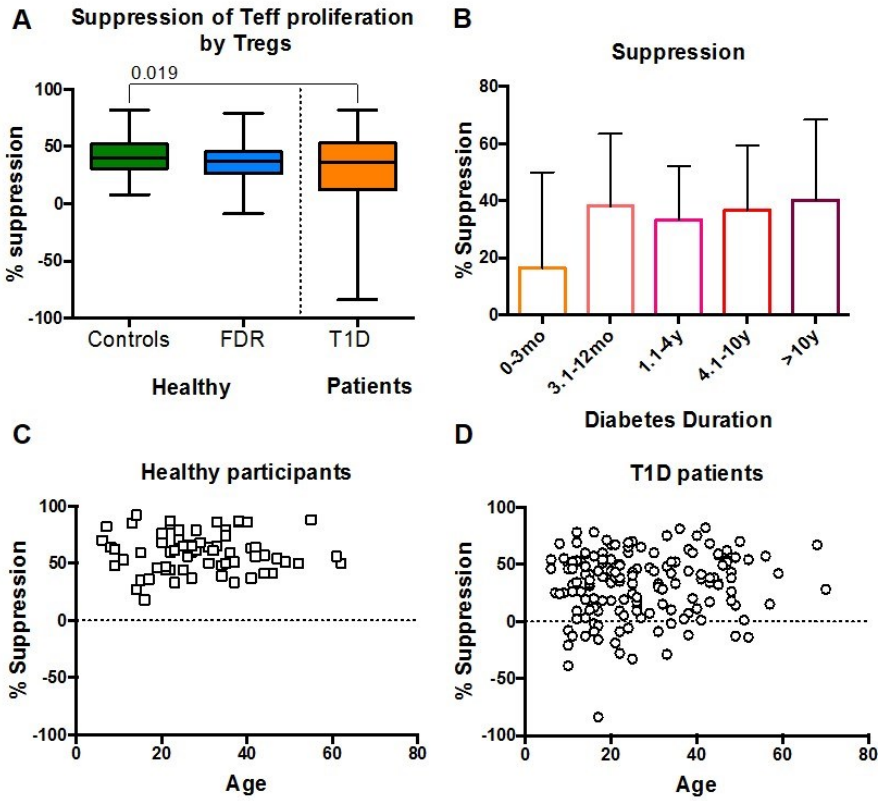

Figure 21: Differences in the suppressive function of $CD4^{pos}$ regulatory T cells (Treg) **A.** Box and whisker plots showing the results for the % of suppression by Treg calculated according to the equation given above for unrelated healthy controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values. p-values are shown on top of the horizontal lines **B.** Bar chart showing the mean % suppression by Tregs from T1D patients with data sorted according to diabetes duration. Data given as mean + SD **C + D** Pearson correlation of age and % suppression for samples from healthy controls (C) and T1D patients (D).

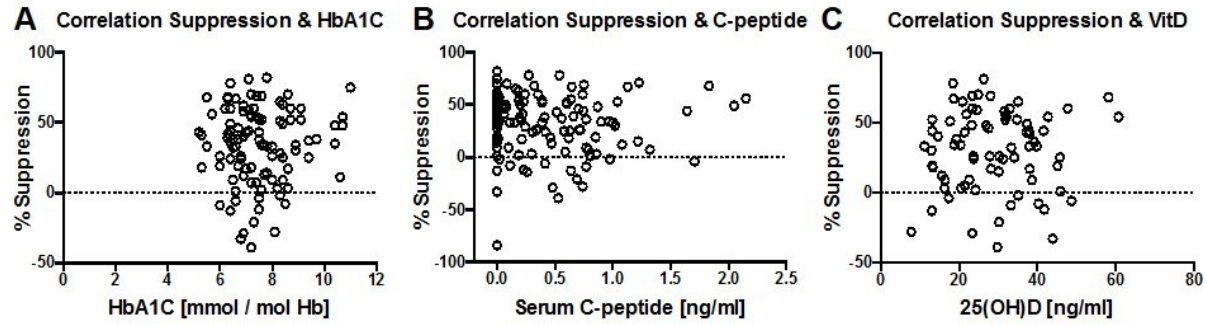

Figure 22: No correlation of % suppression by Tregs and serum C-peptide, HbA1c levels or vitamin D levels in the peripheral blood. **A.** Graph showing the Pearson correlation of the level of HbA1C and % Suppression from T1D patients**. B.** Pearson correlation of the level of serum C-peptide and % Suppression from T1D patients **C.** Pearson correlation of the level of serum vitamin D and % Suppression from T1D patients. Negative values for suppression indicate increased proliferation of effector T cells in the presence of Tregs.

Subsequent analyses, based on age and diabetes duration, confirmed these results. Tregs from newly diagnosed children and adults with T1D had a mean suppressive capacity of 16.41% ± 33.57% which was significant decreased when compared to unrelated healthy, age adjusted controls (39.89% ± 26.19%, p = 0.009; Fig. 23 A). Patients with a diabetes duration not longer than 4 years also showed a decrease in the suppressive capacity of their Tregs (28.18% ± 29.00%) when compared to healthy participants (38.68% ± 20.51%, p = 0.030; Fig. 24 A). Data on Treg suppression from first degree relatives were not significant different to data from unrelated healthy controls, neither in newly diagnosed nor in recently diagnosed cohorts and auto-antibody positivity leaded to no distinct pattern in the distribution of results (Fig. 23 + 24).

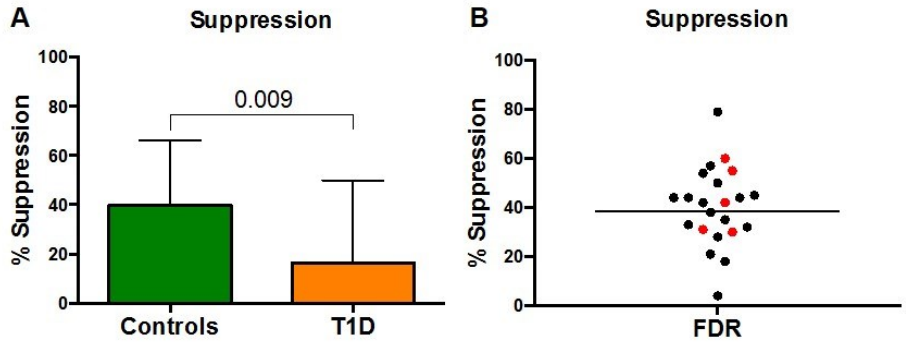

Figure 23: subgroup analysis of impaired suppressive Treg function in newly diagnosed T1D patients (DD < 3months) and age adjusted healthy controls and first degree relatives. **A.** Bar graph showing the results for the % suppression by Treg for unrelated healthy controls and T1D patients (T1D). **B.** Percentage of suppression in samples from first degree relatives (FDR) of the investigated patients. Red dots indicate T1D relevant auto-antibody positivity.

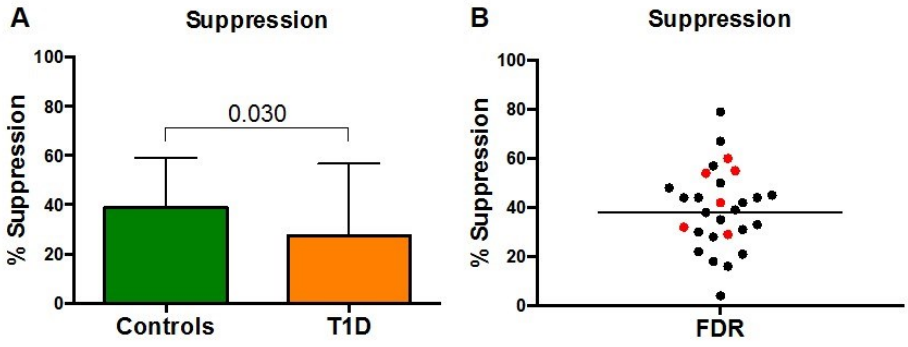

Figure 24: subgroup analysis of impaired suppressive Treg function in recently diagnosed T1D patients (DD < 4 years) and age adjusted healthy controls and first degree relatives. **A.** Bar graph showing the results for the % suppression by Treg for unrelated healthy controls and T1D patients (T1D). **B.** Percentage of suppression in samples from first degree relatives (FDR) of the investigated patients. Red dots indicate T1D relevant auto-antibody positivity.

Tregs from T1D patients show increased apoptosis compared to healthy cells

The percentage of apoptotic cells within FACS sorted Tregs was significantly higher in the T1D group (median + IQR: 1.34% [0.61-2.53]) when compared to healthy controls (0.90% [0.56 – 1.90]; p = 0.031; Fig. 25 A). In contrast to this the % of apoptotic cells within FACS sorted effector T cells (Teffs) was not significant different in any of the investigated groups (healthy controls: 0.87 % [0.38-1.85] versus T1D patients: 1.08 [0.56-1.98]; Fig. 25 B).

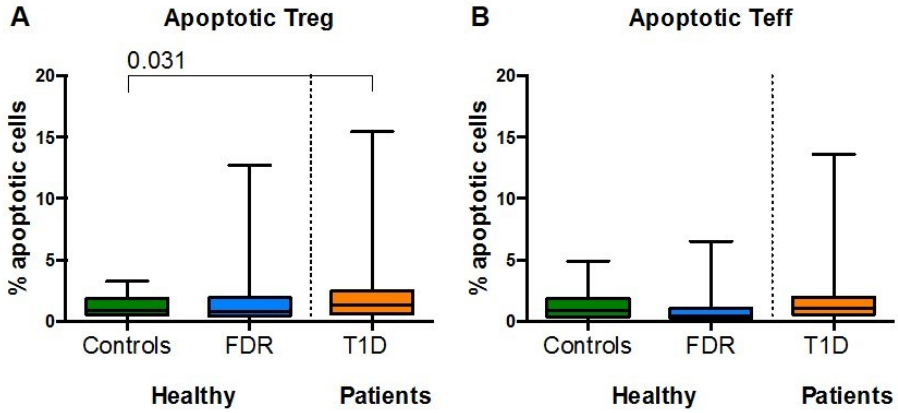

Figure 25: Differences in the percentage of apoptotic cells within Tregs and Teffs. **A.** Box and whisker plots showing the increase in % apoptotic cells within isolated Tregs in T1D patients when compared to healthy unrelated controls. **B.** Box and whisker plots showing the % apoptotic cells within effector T cells (Teff). Data given as median and IQR, whiskers indicating the minimum and maximum values. p-values are shown on top of the horizontal lines.

Subsequent analysis of data in accordance to age and diabetes duration confirmed these results. For example, the % apoptotic cells within Tregs was significantly increased in newly diagnosed T1D patients (2.75 ± 3.05%) when compared to age adjusted healthy controls (0.86 ± 0.79%; p = 0.008; Fig. 26 A). In this subgroup the percentage of apoptotic cells within Teffs was also significantly increased (T1D patients: 1.93 ± 1.76%; healthy controls: 0.84 ± 0.88%, p = 0.006; Fig. 26 C). The percentage of apoptosis within Tregs or Teffs was not different in FDR when compared to unrelated healthy controls (Fig. 26).

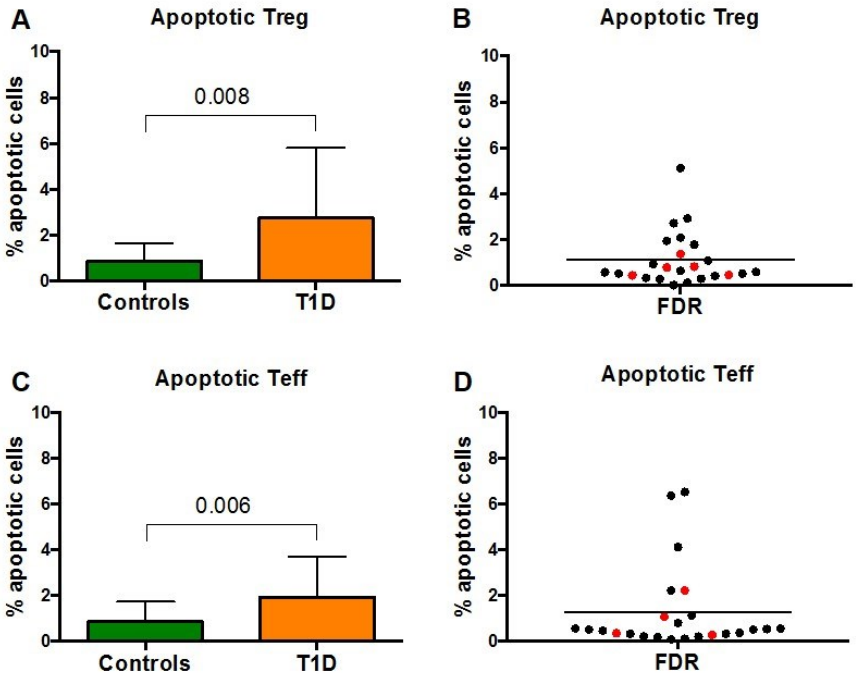

Figure 26: Differences in the percentage of apoptotic cells within Tregs and Teffs in samples from newly diagnosed T1D patients, age-adjusted healthy controls and first degree relatives (FDR) **A.** Box plots showing the increase in % apoptotic cells within isolated Tregs in T1D patients when compared to healthy unrelated controls **B.** Data for % apoptotic Tregs from first degree relatives **C.** Differences in % apoptotic cells within isolated Teffs between healthy controls and T1D patients **D.** Data for % apoptotic Teffs from first degree relatives. Data given in mean ± SD. p-values are given on top of the horizontal lines. Red dots in the plot for the FDR-data indicate T1D relevant auto-antibody positivity.

No differences in % NK and % NKT cells between T1D patients and healthy volunteers

The percentage of natural killer cells (NK) and natural killer T cells (NKT) within lymphocytes was not significantly different in any of the investigated groups (Fig. 27). In detail, NK cells represented a median value of 8.60% [IQR: 5.83-12.62%] of the lymphocyte population in samples from T1D patients, 8.84% [IQR: 6.65-13.68%] in samples from healthy unrelated controls and 8.08% [IQR: 5.94-14.75%] in samples from first degree relatives of the investigated patients (Fig. 27 C). NKT cells represented a median value of 2.86% [IQR: 1.59-6.3%] of the lymphocyte population in samples from T1D patients, 3.92% [IQR: 2.76-5.57%] in samples from healthy unrelated controls and 3.17% [IQR: 2.09-4.68%] in samples from first degree relatives of the investigated patients (Fig. 27 D).

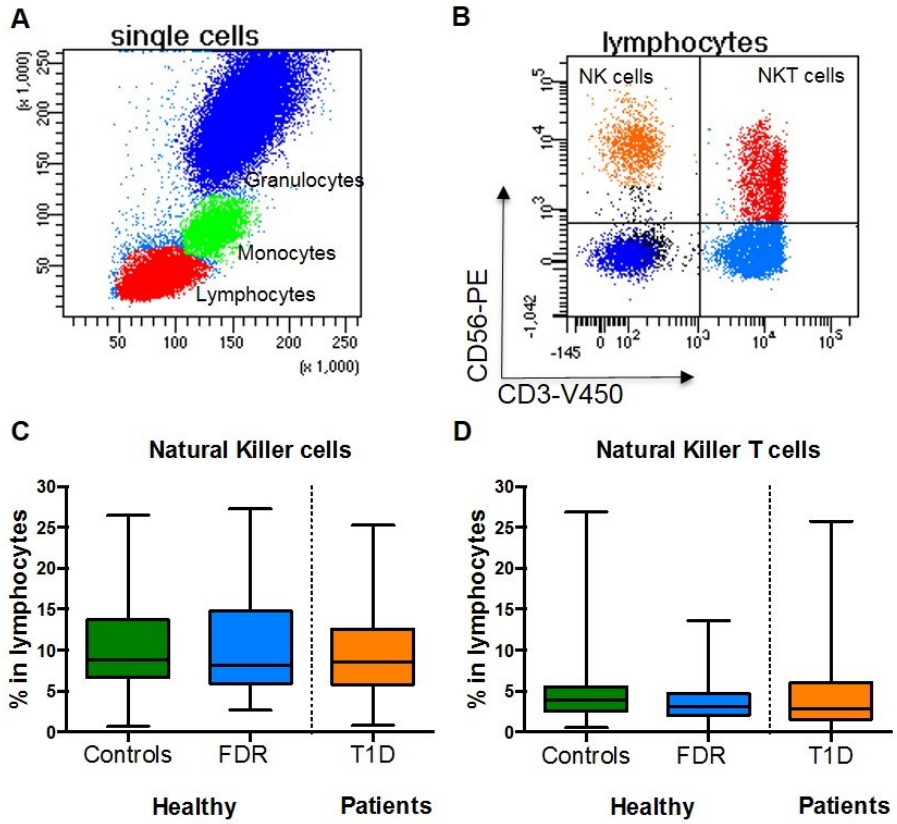

Figure 27: No significant differences in % peripheral natural killer cells (NK) and natural killer T cells (NKT) within lymphocytes. **A.** FACS dot plot showing lymphocytes (red), monocytes (green) and granulocytes (blue) according to their different sizes and granularity. **B.** Gating of $CD3^{neg}CD56^{pos}$ NK cells and $CD3^{pos}CD56^{pos}$ NKT cells within the lymphocyte population. **C. + D.** Box and whisker plots showing the data for NK cells (C.) and NKT cells (D.) from unrelated controls, first degree relatives (FDR) and T1D patients (T1D).Data given as median and IQR, whiskers indicating the minimum and maximum values.

<u>No differences in % pDC and % mDC cells between T1D patients and healthy volunteers</u>

The percentage of plasmacytoid dendritic cells (pDC) and myeloid dendritic cells (mDC) in the peripheral blood was not significantly different in any of the investigated groups (Fig. 28). In detail, pDCs represented a median value of 36.81% [IQR: 30.74-44.40%] of the $linage^{neg}HLADR^{pos}$ lymphocyte population in samples from T1D patients, 31.80% [IQR: 24.84-39.31%] in samples from healthy unrelated controls and 37.09% [IQR: 28.20-45.83%] in samples from first degree relatives of the investigated patients. Myeloid dendritic cells represented a median value of 50.12% [IQR: 41.61-55.16%] of the $linage^{neg}HLADR^{pos}$ lymphocyte population in samples from T1D patients, 48.04% [IQR: 41.81-57.60%] in samples from healthy unrelated controls and 49.43% [IQR: 42.99-57.51%] in samples from first degree relatives of the investigated patients.

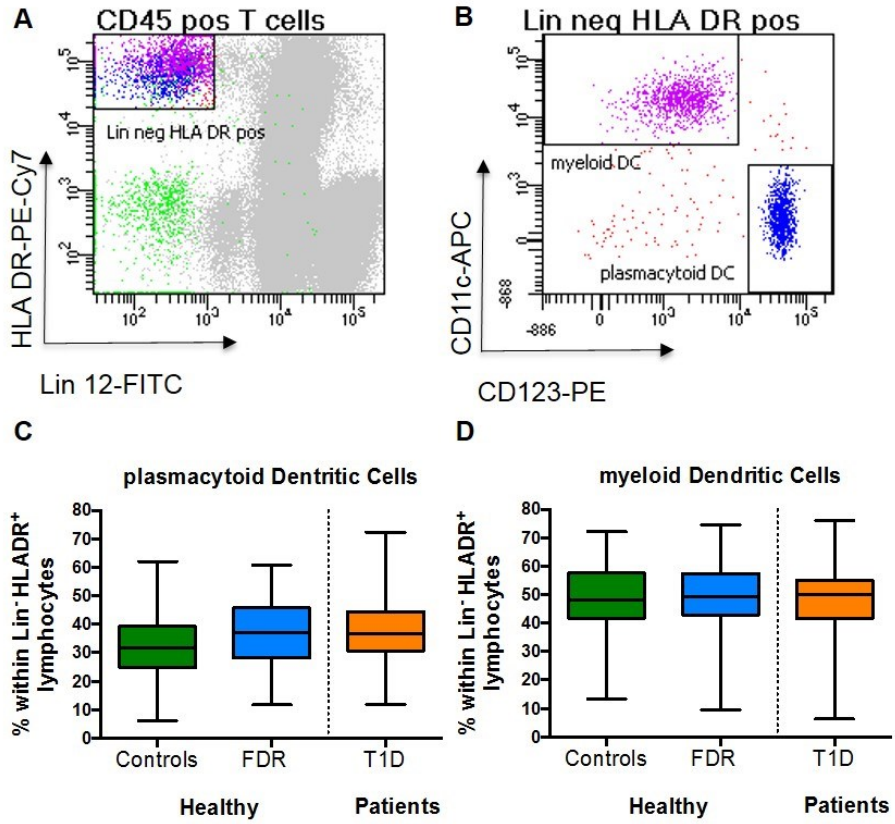

Figure 28: Peripheral plasmacytoid and myeloid dendritic cells (pDC and mDC). **A.** Gating of $HLADR^{pos}Lin^{neg}$ cells within leukocytes **B.** Gating of myeloid DC (mDC) and plasmacytoid DC (pDC) within $HLADR^{pos}Lin^{neg}$ cells **C.+D.** Box and whisker plots showing the data for pDC (C.) and mDC (D) from unrelated controls, first degree relatives (FDR) and T1D patients (T1D). Data given as median and IQR, whiskers indicating the minimum and maximum values.

Differences in the expression of GLP1-receptor in peripheral immune cells of healthy volunteers and T1D patients

Human peripheral immune cells express Glucagon-Like-Peptide-1 receptor

Study ID: ADPP001 subgroup analysis

The expression of the GLP-1 receptor (GLP-1R) was quantified in peripheral B-cells, monocytes, granulocytes and T-cell subtypes, including Tregs, from healthy donors (n=13), newly diagnosed T1D patients (n=10) and longstanding T1D patients (n=13) by FACS technology. Demographics are shown in table 7.

GLP1-R staining	Healthy controls	Newly diagn. T1D	Longst. T1D
n	13	10	13
Females (%)	46	50	38
Age (yrs)	23.3 ± 8.2	21.4 ± 12.7	28.2 ± 11.9
BMI (kg/m^2)	22 ± 8	20 ± 7	21 ± 8
T1D duration (years)	--	0.2 [0.1-0.2]	5.7 [3.4-8.7]
NLR	1.13 ± 0.41	1.39 ± 0.48	1.58 ± 0.53
CD4/CD8 ratio	2.2 ± 1.2	1.8 ± 0.46	1.8 ± 0.99
Treg (% of CD4)	5.1 ± 2.7	4.6 ± 2.2	4.3 ± 1.5

Table 7: Demographics of all participants included in the subgroup analysis for GLP-1 receptor staining. BMI: body mass index; NLR: neutrophil to lymphocyte ratio; normally distributed data are given in mean ± SD, not normally distributed data are given in median (+ interquartile range).

The staining of HEK293-cells (Human Embryonic Kidney Cell Line), that per definition do not express GLP1-R, served as a negative control (Fig. 29). For the appropriate definition of positive signals, the Fluorochrome-Minus-One (FMO) method, isotype controls and unstained samples where used (Fig. 29).

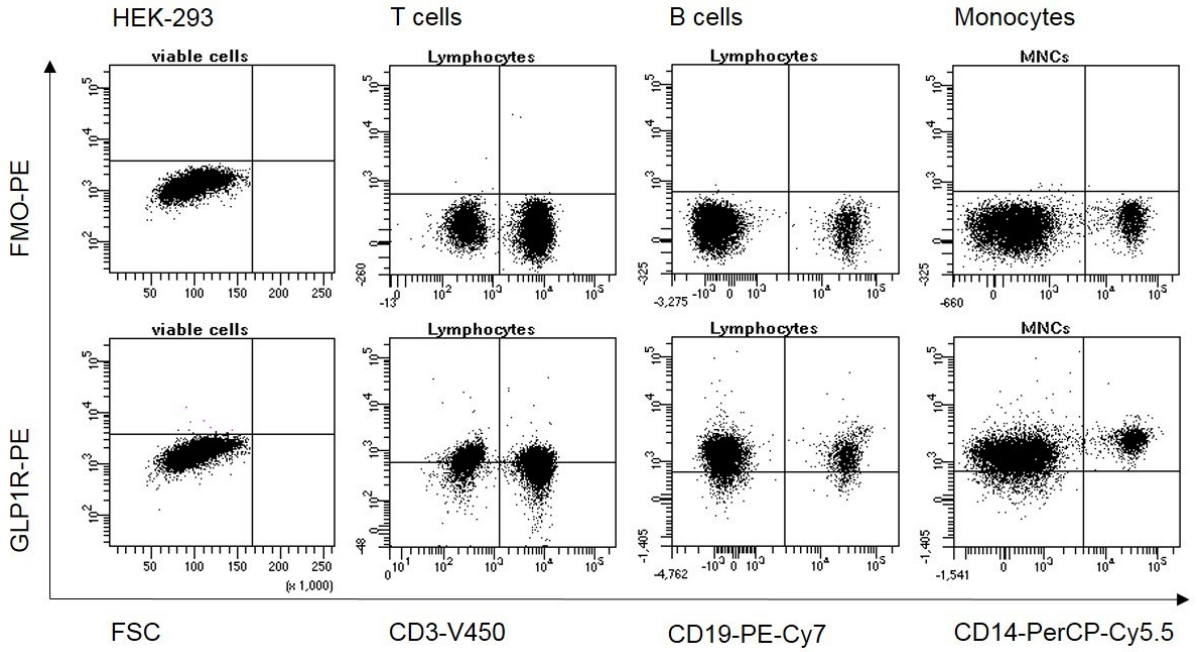

Figure 29: Flow cytometry dot plots showing representative data from intracellular staining of the GLP1-R in HEK-293 cells (negative control), T cells, B cells and monocytes. Gates were determined by fluorescence Minus One controls (FMOs, shown in the upper line) and unstained samples and isotype controls (not shown). Plots for positive signals are shown below the corresponding FMO plots.

All investigated human peripheral immune cell types from healthy participants expressed the GLP1-R. Monocytes showed the highest GLP1-R expression with a mean of 96.06 ± 4.66% (SD), $CD19^{pos}$ B cells had the second highest expression (81.52 ± 15.48%) and 53.80 ± 24.89% of $CD3^{pos}$ T cells were positive for the GLP1-R (Fig. 30 A). Validation of FACS quantification was done with RT-PCR. Therefore, purchased pure mRNA from healthy controls was quantified for the expression of GLP1-R-mRNA. As shown in figure 30 the same distribution as with FACS analysis was found with the mRNA quantification.

In the $CD4^{pos}$ as well as in the $CD8^{pos}$ T- cell population from healthy participants, significantly more cells with memory phenotype ($CD45RA^{neg}CD45RO^{pos}$) expressed GLP1-R than those with a naive phenotype ($CD45RA^{pos}CD45RO^{neg}$) (Fig. 30 C+D). In detail, 51.91 ± 27.18 % of naive $CD4^{pos}$ T helper cells from healthy participants were positive for GLP1-R staining, whereas 72.13 ± 19.01 % of the memory $CD4^{pos}$ T helper cells showed a positive GLP1-R staining signal (p=0.046). For cytotoxic $CD8^{pos}$ T cells, 60.30 ± 24.67 % of the naive cells were positive for GLP1-R and 89.98 ± 9.73 % of the memory cells showed GLP1R expression (p<0.001).

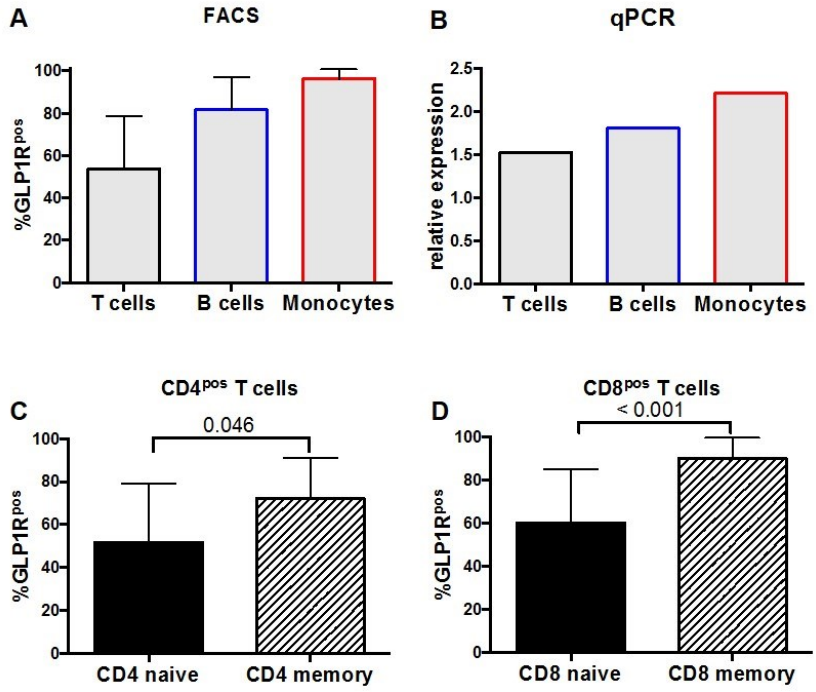

Figure 30: Quantification of GLP1-R expression in human peripheral immune cells. **A** analysis of GLP1-R^{pos} T-cells, B-cells and monocytes in healthy humans by flow cytometry. **B** Validation of FACS results by real-time PCR with purchased human RNA from sorted immune cells donated by healthy volunteers. **C** GLP1-R expression in subtypes of $CD4^{pos}$ T helper cells from healthy donors. **D** GLP1-R expression in subtypes of $CD8^{pos}$ T helper cells from healthy humans. Data given as mean ± SD. P-values are given on top of the horizontal lines.

Differences in GLP1-receptor expression between healthy volunteers and T1D patients

Both, healthy and newly diagnosed T1D samples, showed significantly more GLP1-R^{pos} cells within the memory subtype of $CD4^{pos}$ helper- and $CD8^{pos}$ cytotoxic-T cells. In contrast to this, the expression of GLP1-R on $CD4^{pos}$ and $CD8^{pos}$ T cells from long-term T1D patients was not significantly different (Fig. 31 A + B). This resulted from a significant increase in the proportion of GLP1-R^{pos} naive $CD4^{pos}$ T cells in long-term T1D patients (78.01 ± 18.99%) when compared to healthy controls (51.91 ± 27.18%; adjusted p-value = 0.031) or newly diagnosed T1D patients (38.85 ± 25.03%; adjusted p-value = 0.002). Likewise the percentage of GLP1-R^{pos} memory CD4pos T cells was significantly increased in long-term T1D patients (86.11 ± 14.37%) when compared to newly diagnosed T1D patients (62.66 ± 19.37%; adjusted p-value = 0.014). (Fig. 31 A). Naive cytotoxic $CD8^{pos}$ Tc from long-term T1D patients also showed an increased proportion of GLP1-R^{pos} signals (85.39 ± 16.78%) when compared to healthy controls (60.30 ± 24.67%; p=0.034) or newly diagnosed T1D patients (58.60 ± 29.37%; adjusted p=0.031; Fig. 31 B). Memory $CD8^{pos}$ cytotoxic T cells were not significantly different in any of the investigated groups.

In regulatory T cells (Tregs), a significant increase in the % GLP1-R^{pos} cells was found in long-term T1D patients (72.11 ± 18.05%) when compared to newly diagnosed T1D patients (45.78 ± 18.91%; p=0.006; Fig. 31 C). Furthermore, GLP1-R expression was found to be increased and $CD19^{pos}$ B cells from long-term T1D patients (93.00 ± 5.58 %) when compared to cells from newly diagnosed T1D patients (71.85 ± 20.95%, p=0.008; Fig. 31 D). Finally, the percentage of GLP1-R^{pos} granulocytes and monocytes was not significantly different in any of the investigated groups (Fig. 31 E + F) and percentage of GLP1-R^{pos} cells was highest in monocytes (Fig. 31 F).

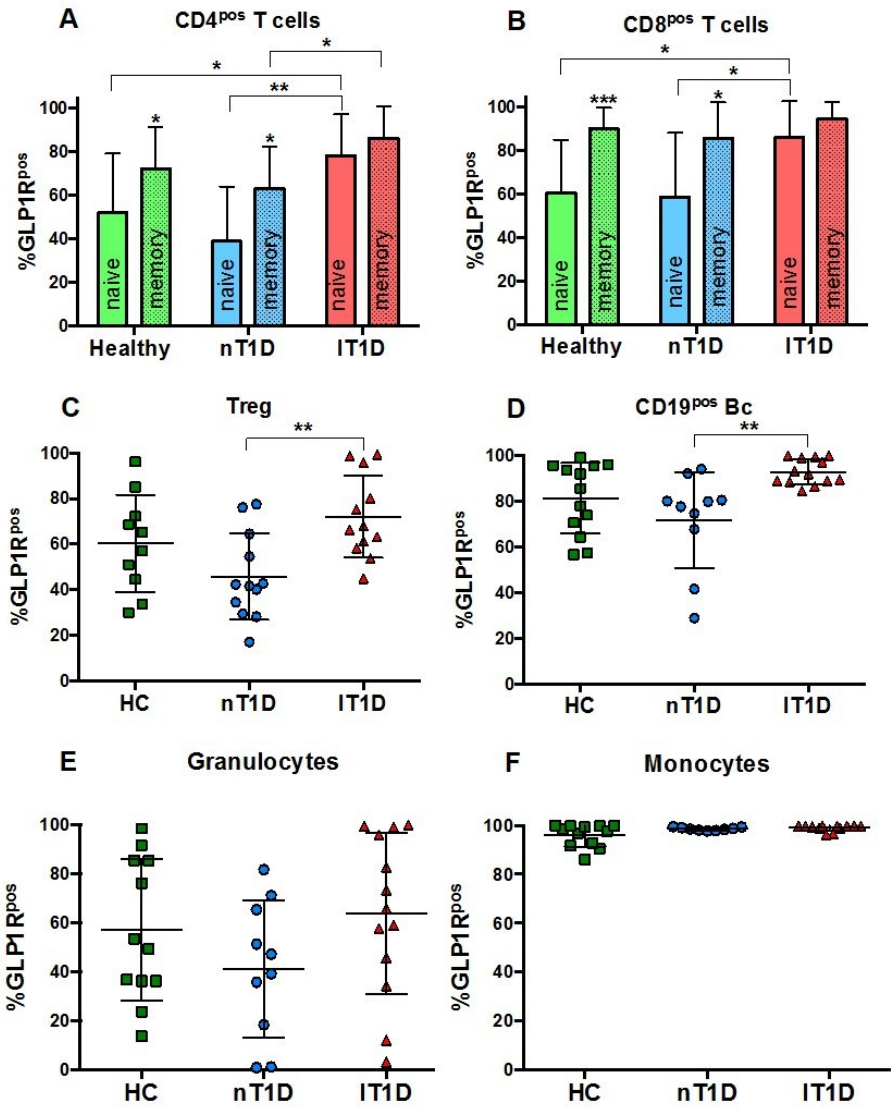

Figure 31: Quantification of GLP1-R expression in peripheral immune cells from healthy controls (HC, green), newly diagnosed T1D patients (nT1D, blue) and longstanding T1D patients (lT1D, red). **A.** GLP1-R expression in naive (blank boxes) and memory (dotted boxes) $CD4^{pos}$ T cells. **B.** GLP1-R expression in naive (blank boxes) and memory (dotted boxes) $CD8^{pos}$ T cells. **C.** GLP1-R expression in regulatory T cells. **D.** GLP1-R expression in $CD19^{pos}$ B cells. **E.** GLP1-R expression in granulocytes. **F.** GLP1-R expression in monocytic cells. Data given as mean +/- SD. Significant p-values are indicated by * above a horizontal line. * $p<0.05$, ** $p<0.01$.

Differences in the distribution of T cell subtypes throughout the human intestinal tract

Study ID: ADPP003 baseline

In total 16 healthy young adults were included in this trial. All participants underwent a gastro-duodenoscopy and, on the following day, a colonoscopy. All 16 participants completed the baseline visits (see adapted Consort flow diagram Fig. 9). Baseline demographics are shown in table 8.

ADPP003 Baseline	
n	16
Females (%)	44
Age (yrs)	25 ± 4
Height (cm)	172 ± 8
Weight (kg)	69 ± 11
BMI (kg/m^2)	23 ± 3
Leukocytes (G/L)	6.2 ± 1.3
Serum C-reactive protein (mg/L)	3.8 ± 9.2
Serum 25(OH)D (ng/ml)	22.3 ± 13.1
NLR	1.3 ± 0.5
CD4/CD8 ratio	2.0 ±1.1
Treg (% of CD4)	6.2 ± 2.5

Table 8: Demographics and clinical baseline parameter of all participants included in the ADPP003 vitamin D supplementation study. BMI: body mass index, 25(OH)D: 25-hydroxyvitamin D, NLR: neutrophile to lymphocyte ratio; Data are given in mean ± SD. G/L: giga/l = 10^9/l blood

Differences in the distribution of distinct T cell subtypes were found in isolated cells from tissue biopsies taken from seven different regions throughout the whole human intestinal tract (Fig. 32).

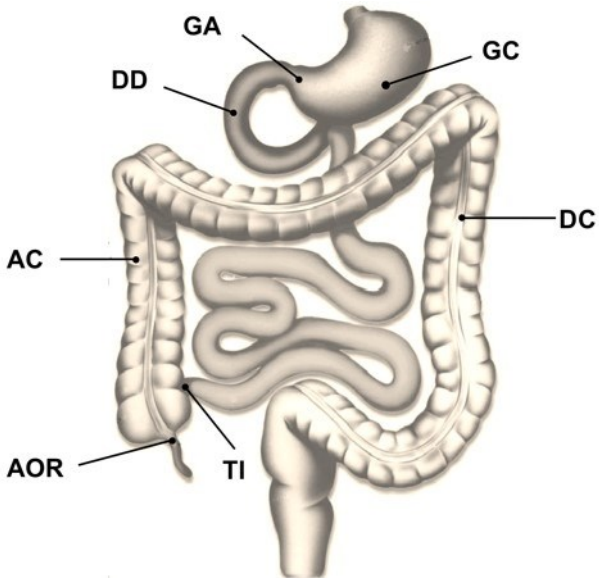

Figure 32: Schematic overview of biopsy regions. GC: gastric corpus region, GA: gastric antrum region, DD: duodenum, TI: terminal ileum region, AOR: appendiceal orifice region, AC: ascending colon region at 10 cm distal to the ileocecal valve; SC: sigmoid colon region at 30 cm proximal to the anal canal. Adapted with permission from Tauschmann et al. (75).

T cells, defined by expression of surface CD3 molecules, were more or less consistently found throughout the whole human intestinal tract (table 9; Fig. 33 B). The percentage of $CD8^{pos}$ cytotoxic T cells within $CD3^{pos}$ cells was significantly increased in the gastric mucosa, particularly in the gastric corpus relative to the duodenum and large intestine regions (tables 9 + 10; Fig. 33 D). In contrast to this, the percentage of $CD4^{pos}$ T helper cells within $CD3^{pos}$ cells was increased along the lower gastrointestinal tract when compared to the gastric regions. Lowest levels of $CD4^{pos}$ T helper cells were found in the gastric antrum and the gastric corpus whereas the maximal levels were found in the sigmoid colon (tables 9 + 10; Fig. 33 F). Additionally, $CD4^{pos}$ T helper cells outweighed $CD8^{pos}$ cytotoxic T cells throughout all regions of the small and large intestine, as indicated by a CD4 to CD8 ratio above 1.00 (see tables 9 + 10). Regulatory T cells were increased in the appendiceal orifice region and the ascending colon compared to both, the gastric region as well as the duodenal mucosa. Furthermore, a significant difference between the percentage of Tregs between the AO region and the terminal ileum was found (table 10, Fig. 34 B). Within Tregs the percentage of Helios positive cells was consistently distributed throughout the whole gastrointestinal tract (Fig. 34 D).

Upper GI tract	GC	GA	DD
$CD3^{pos}$ (% of lymphocytes)	69.6 [54.0-74.5]	53.3 [38.0-63.0]	51.8 [44.0-61.8]
$CD8^{pos}$ (% of lymphocytes)	39.7 [32.6-52.9]	30.3 [22.6-43.3]	17.8 [8.0-25.4]
$CD4^{pos}$ (% of lymphocytes)	17.7 [13.8-27.4]	12.5 [9.1-21.8]	36.1 [31.6-41.5]
CD4/CD8 ratio	0.4 [0.3-0.7]	0.4 [0.3-0.8]	2.0 [1.3-4.3]
Treg (% of CD4)	2.0 [1.4-3.3]	2.1 [1.2-3.4]	1.6 [1.0-3.2]

Table 9: T cell subclasses and relative abundances in the upper gastro-intestinal tract. N = 16; Data are given in median (IQR). GC: gastric corpus region, GA: gastric antrum region, DD: duodenum; With permission from PlosONE (75).

Lower GI tract	TI	AO	AC	DC
$CD3^{pos}$ (% of lymphocytes)	59.1 [54.5-70.6]	59.4 [53.0-66.9]	60.7 [48.4-64.4]	59.3 [53.0-66.3]
$CD8^{pos}$ (% of lymphocytes)	13.2 [9.9-31.0]	13.0 [11.6-16.7]	12.2 [9.2-17.7]	12.1 [8.7-17.5]
$CD4^{pos}$ (% of lymphocytes)	39.9 [35.1-45.4]	38.2 [33.9-46.7]	37.0 [34.6-44.2]	41.2 [37.3-46.2]
CD4/CD8 ratio	3.0 [1.3-4.1]	3.1 [2.5-3.4]	3.2 [2.0-3.9]	3.0 [2.4-5.0]
Treg (% of CD4)	3.0 [2.5-4.6]	4.6 [3.3-5.7]	4.0 [3.2-5.9]	3.5 [2.2-5.2]

Table 10: T cell subclasses and relative abundances in the lower intestinal tract. N = 16; Data are given in median (IQR). TI: terminal ileum region, AOR: appendiceal orifice region, AC: ascending colon region, SC: sigmoid colon region. With permission from PlosONE (75).

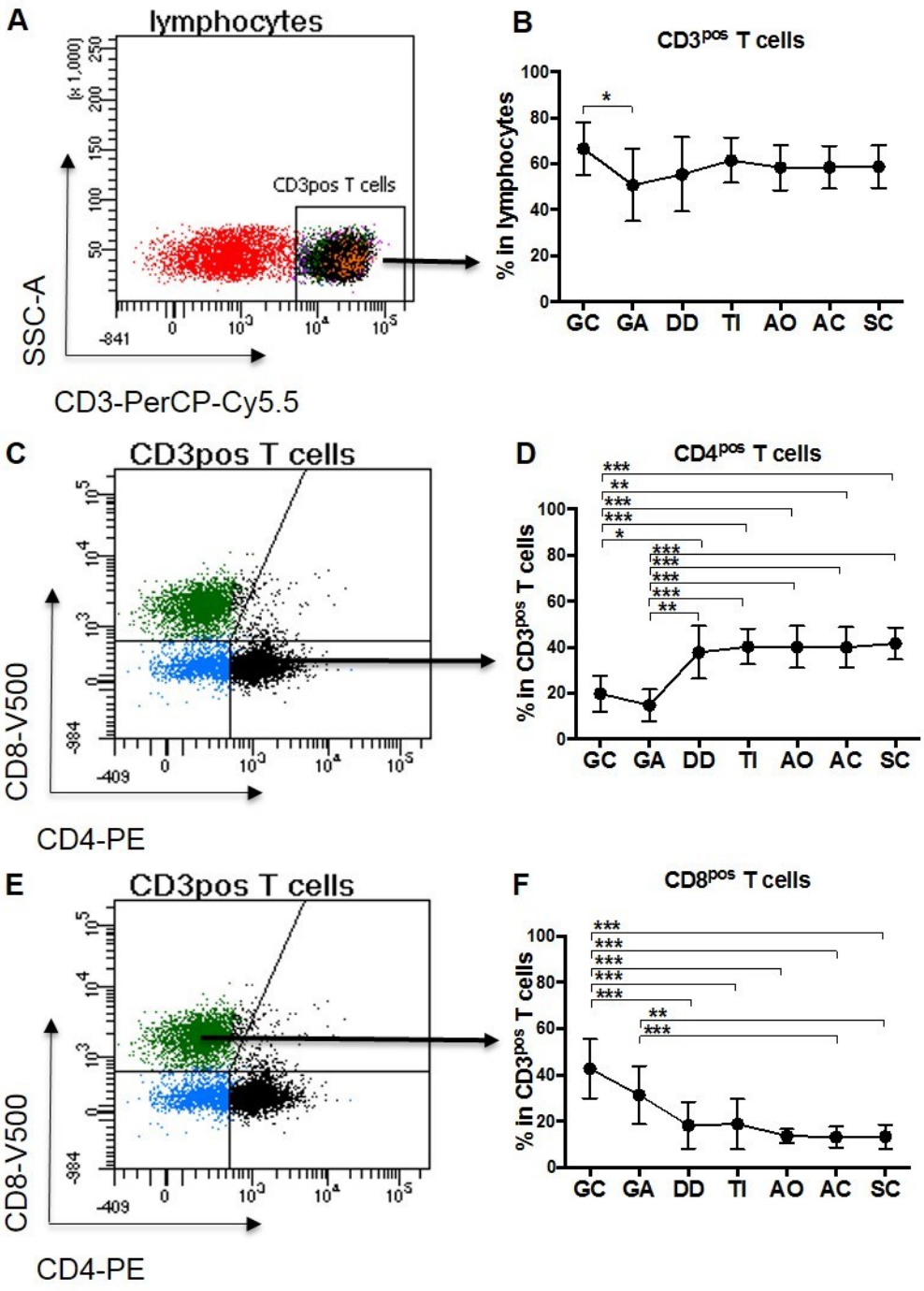

Figure 33: Significant different percentages of T cells and T cell subtypes in the investigated regions of the intestinal mucosa. **A.** FACS gating strategy for CD3pos T cells **B.** $CD3^{pos}$ T cells are more or less evenly distributed throughout the whole gastro-intestinal compartment. **C.** FACS gating strategy for $CD4^{pos}$ helper T cells **D.** $CD4^{pos}$ helper T cells are predominantly enriched in the lower intestinal tract **E.** FACS gating strategy for $CD8^{pos}$ cytotoxic T cells **F.** $CD8^{pos}$ cytotoxic T cells are mostly enriched in the upper gastro-intestinal tract. GC: gastric corpus region, GA: gastric antrum region, DD: duodenum, TI: terminal ileum region, AOR: appendiceal orifice region, AC: ascending colon region, SC: sigmoid colon region. Adapted with permission from PlosONE (75).

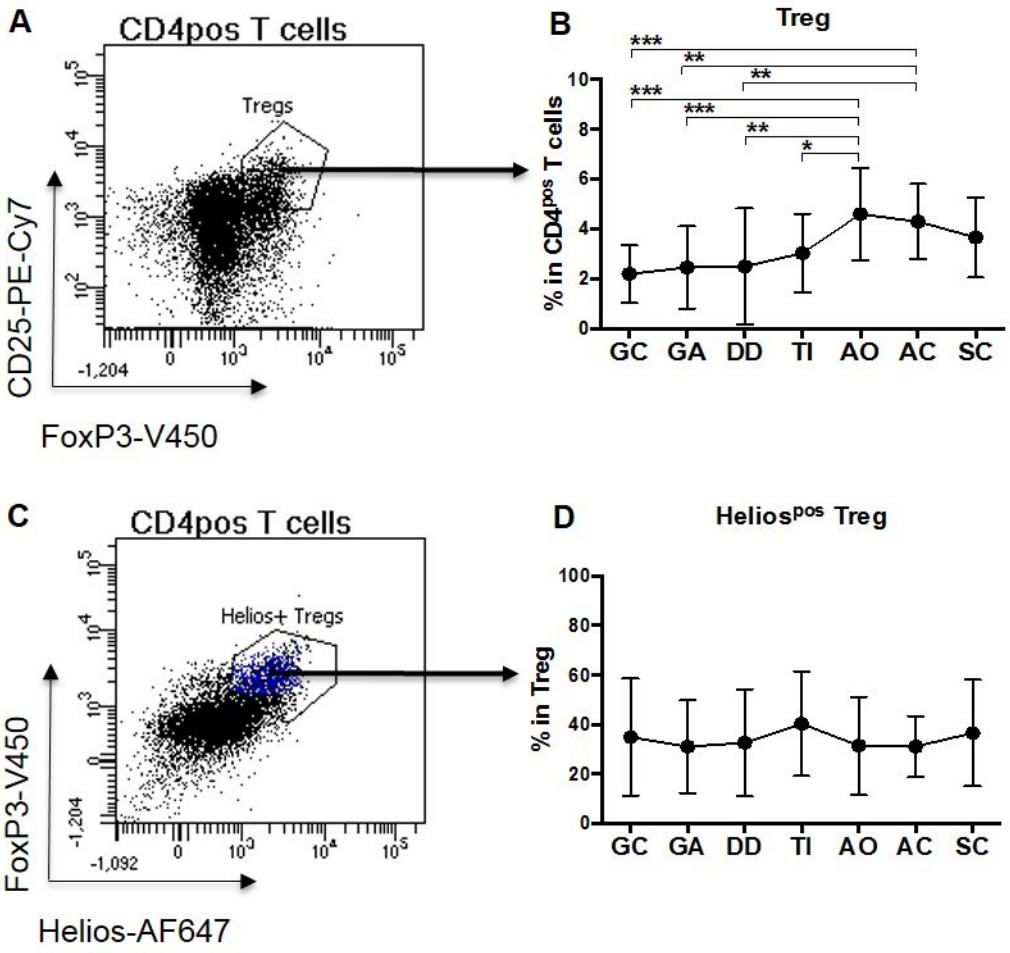

Figure 34: Differences in the percentages of regulatory T cells (Tregs) and Helios expression within Tregs in the investigated regions of the intestinal mucosa. **A.** FACS gating strategy for $CD4^{pos}CD25^{hi}FoxP3^{pos}CD127^{neg}$ Tregs **B.** The percentage of Tregs is highest in the appendiceal orifice region and the ascending colon **C.** FACS gating strategy for $Helios^{pos}$ Tregs **D.** $Helios^{pos}$ Tregs are evenly distributed throughout the whole gastro-intestinal region. GC: gastric corpus region, GA: gastric antrum region, DD: duodenum, TI: terminal ileum region, AOR: appendiceal orifice region, AC: ascending colon region, SC: sigmoid colon region. Adapted with permission from PlosONE (75).

Effects of cholecalciferol supplementation on the percentage of immune cell subtypes in the peripheral blood and intestinal tissue

Cholecalciferol supplementation increases serum 25(OH)D levels and calcitriol levels without causing side effects

Study IDs: Healthy VitD, ADPP002, ADPP003

At baseline, 69% of our healthy study participants participating in the healthy VitD study, 75% of the healthy participants enrolled in the ADPP003 study and 63% of the newly diagnosed T1D patients recruited for the ADPP002 study had serum 25(OH)D levels in the vitamin D deficient (<20ng/dl) or insufficient (20-30ng/dl) range. Baseline demographics for the healthy VitD study and the T1D study (ADPP002) are shown in table 11. Baseline demographics for healthy participants investigated in the ADDPP003 study are shown in table 8.

Healthy VitD study	vitD group	placebo group	p value
n	30	29	
Gender (% female)	47	52	
BMI (kg/m^2)	24.5 ± 3.9	23.9 ± 3.4	0.625
Age (years)	27 [24-37]	35 [26-42]	0.192
Serum 25(OH)D (ng/ml)	22.4 [16.8-31.6]	26.0 [17.7-32.0]	0.644
NLR	1.41 [1.03-1.86]	1.42 [1.26-1.69]	0.836
CD4/CD8 ratio	1.87 [1.53-2.74]	2.09 [1.49-2.82]	0.703
Tregs in CD4 (%)	4.9 ± 0.9	5.3 ± 1.0	0.075

Newly diagnosed T1D / ADPP 002	vitD group	placebo group	p value
n	14	15	
Gender (% female)	21	27	
BMI (kg/m^2)	18.1 [16.4-21.6]	17.1 [15.6-18.1]	0.141
Age (years)	12 [11.0-17.5]	13 [9.5-15.5]	0.584
Diabetes duration (days)	61 ± 20	61 ± 28	0.991
HbA1c (mmol/mol)	54.5 ± 11.6	63.5 ± 16.1	0.104
Serum 25(OH)D (ng/ml)	26 [14.6-29.9]	23.6 [20.4-41]	0.143
NLR	1.28 [0.96-1.53]	1.33 [1.17-1.75]	0.713
CD4/CD8 ratio	1.74 [1.17-1.91]	1.55 [1.31-1.94]	0.626
Tregs in CD4 (%)	6.1 ± 1.9	4.8 ± 1.7	0.081

Table 11: Demographic data of our studied populations at time of recruitment. Data are presented as mean ± SD when normally distributed or median + (IQR) when not normally distributed. NLR = neutrophile to lymphocyte ratio.

In healthy individuals participating in the healthy VitD study serum 25(OH)D levels increased significantly in the vitD group (25.5 ± 11.4 at baseline vs. 55.1 ± 18.1 ng/ml after 12 weeks, p < 0.001) whereas it decreased significantly in the placebo group (25.8 ± 10.4 at baseline vs. 21.1 ± 9.8 ng/ml after 12 weeks; p = 0.003, Fig. 35 A). Additionally, measured serum calcitriol levels were similar between the vitD and placebo group at baseline (74.1 ± 38.1 vs. 75.3 ± 37.1 pmol/ml). After 12 weeks of supplementation, the concentration of calcitriol changed significantly only in the healthy vitD group (100.1 ± 40.7 pmol/ml, p = 0.016) but remained stable in the healthy placebo group (78.3 ± 47.5 pmol/ ml, p = 0.7; Fig. 35 B).

In healthy individuals participating in the ADPP003 study, serum 25(OH)D levels increased significantly from 22.3 ± 13.1 ng/ml at baseline to 67.3 ± 13.7 ng/ml after 8 weeks of cholecalciferol supplementation (p < 0.001). After the termination of cholecalciferol treatment serum 25(OH)D levels decreased again and reached a final level of 55.2 ± 13.2 ng/ml at the follow up visit after 10 weeks (p=0.040; Fig. 35 C).

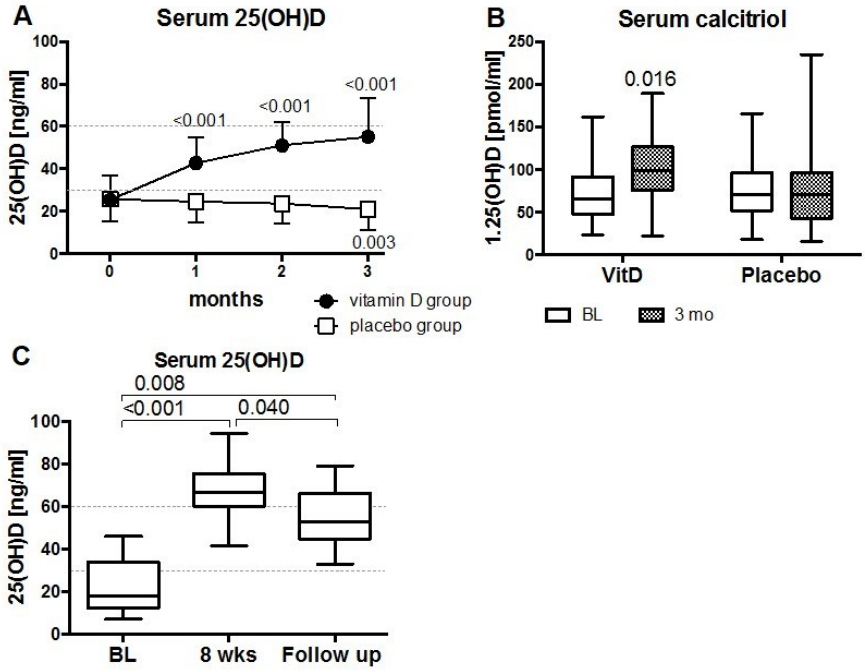

Figure 35: Serum vitamin D levels in healthy individuals participating in two cholecalciferol supplementation trials. **A.** Mean serum 25(OH)D concentrations in healthy participants during the 12-week study period in the vitD group (black dots, n = 30) and the placebo group (white squares, n = 29). **B.** Serum calcitriol levels in healthy participants during the 12-week study period **C.** Serum 25(OH)D concentrations in healthy participants (n=16) during an 8-week vitamin D supplementation and 4 weeks after the last vitamin D intake. Normally distributed data are given in mean ± SD, not normally distributed data are given in median and IQR, p-values are given within the graphs. Dotted lines indicate normal ranges. Adapted with permission from the European Journal of Nutrition (76).

In newly diagnosed T1D patients participating in the ADPP002 study, serum 25(OH)D levels increased significantly after 3 months in the vitD group and remained higher than the levels in the placebo group during the whole treatment period ($p<0.001$; Fig. 36).

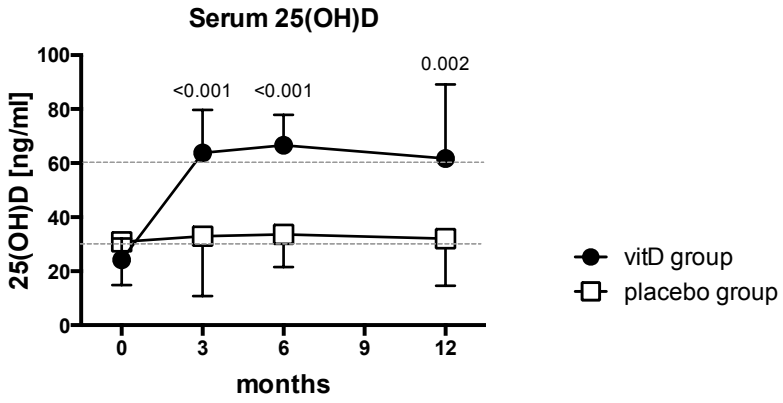

Figure 36: Serum vitamin D levels in newly diagnosed T1D patients participating in a cholecalciferol supplementation trial. Mean serum 25(OH)D concentrations during the 12-months study period in the vitD group (black dots, n = 14) and the placebo group (white squares, n = 15). Data are given in mean ± SD, p-values are given within the graphs. Dotted lines indicate normal ranges. Adapted with permission from Clinical Immunology (77).

In all healthy volunteers as well as in newly diagnosed T1D patients participating in the randomized controlled studies serum calcium levels and urinary calcium/ creatinine ratio were similar in the vitD and placebo group during the whole study period and stayed within the normal ranges in all participants (Fig. 37). Other safety determinants (urine calcium, complete blood cell count, serum phosphorus, serum albumin, PTH and CRP) remained within normal reference ranges during the whole study period in all investigated study participants (data not shown). One patient in the T1D placebo group experienced a marked increase in urinary calcium/ creatinine ratio with normal serum calcium levels and serum 25(OH)D levels, prompting in a 50% dose decrease of study medication and further follow up with renal ultrasound.

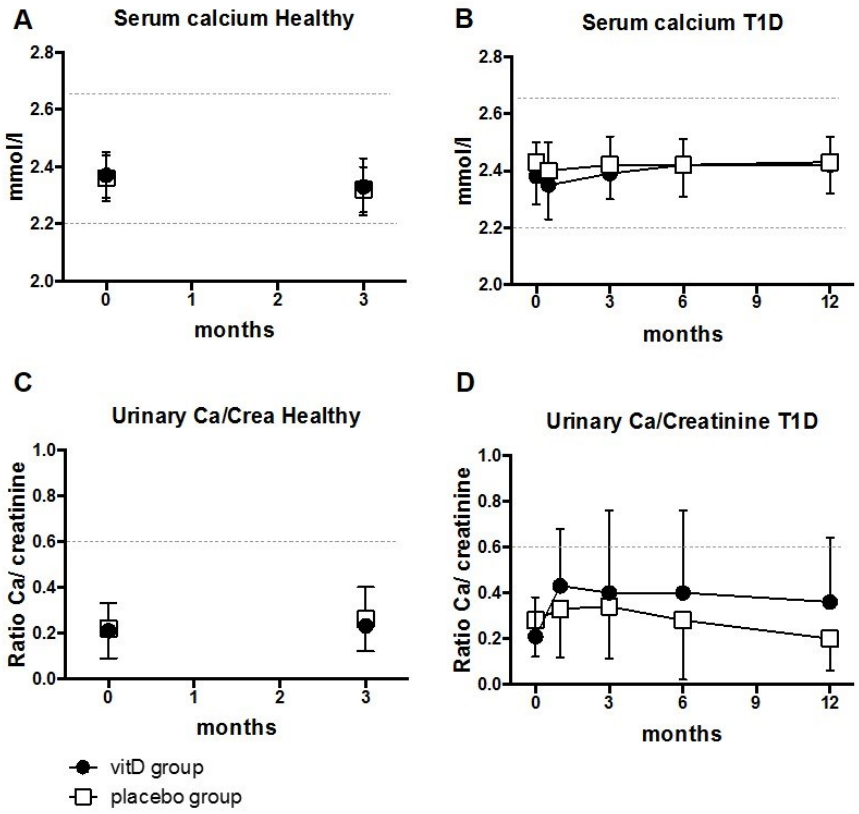

Figure 37: Serum and urinary calcium levels in healthy participants and T1D patients participating in two cholecalciferol supplementation trials. **A** Mean serum calcium concentrations in healthy individuals at baseline and after 12 weeks **B.** Mean serum calcium concentrations in newly diagnosed T1D patients during the 1 year study period **C.** Mean ratio of urinary calcium/ creatinine in healthy participants at baseline and after 12 weeks **D.** Mean ratio of urinary calcium/ creatinine in newly diagnosed T1D patients during the 1 year study period. Data for the vitamin D groups are shown as black dots, data for the placebo groups are given as white squares. Dotted grey lines indicate normal ranges. Adapted with permission from Clinical Immunology (77).

<u>Effects of cholecalciferol on fasting and stimulated C-peptide, HbA1c and daily insulin doses in T1D patients</u>

Endogenous insulin production, measured by fasting and stimulated C-peptide during the mixed meal tolerance test (MMTT), showed no statistical significant difference between the vitD and placebo group during the 12-month study period. However, fasting C-peptide appeared to decrease slower in the vitD group in comparison to the placebo group (p=0.078) (Fig. 38). For stimulated C-peptide the % change of the mean AUC from baseline to month 6 and month 12 showed a similar decline in both groups (Fig. 38 B). Two patients from the vitD group and one patient from the placebo group maintained their AUC C-peptide above baseline values at 6 month but only one patient from the vitD group showed this high level after 12 months of

supplementation. Insulin requirements (calculated per kg bodyweight per day) significantly increased in the placebo group after 12 months compared to baseline ($p<0.001$) but did not significantly change in the vitD group (Fig. 38 C). HbA1c levels were similar between the vitD and the placebo group throughout the whole study period (Fig. 38 D).

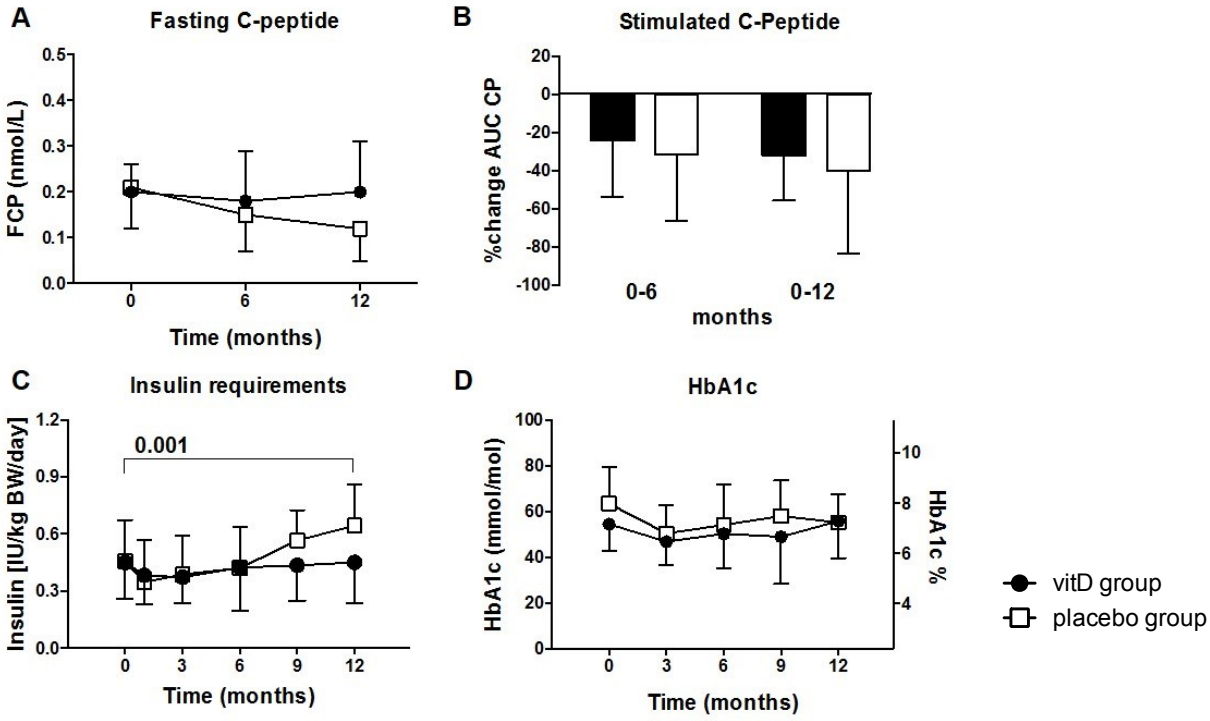

Figure 38: Effects of cholecalciferol supplementation on metabolic parameters in newly diagnosed T1D patients. **A.** Decline of fasting C-peptide in the vitD group (black dots, n=14) and in the placebo group (white squares, n=15) during the 12-months study period. **B.** Changes in the AUC of stimulated C-peptide from baseline to month 6 and month 12 in the vitD group (black bars) and the placebo group (white bars). **C.** Insulin doses calculated per kg bodyweight per day in the vitD group (black dots) and the placebo group (white squares). p-values indicate changes as compared to baseline. **D.** HbA1C levels during the 12 months study period in the vitD group (black dots) and the placebo group (white squares). Data given as mean ± SD. Adapted with permission from Clinical Immunology (77).

<u>Cholecalciferol supplementation changes the percentage of CD4^{pos} Tregs in the peripheral blood of healthy participants but not in T1D patients</u>

Study IDs: Healthy VitD, ADPP002,

In the healthy VitD study the mean percentage of Tregs (%Tregs) within 20 000 CD4^{pos} T cells increased significantly in participants receiving a cholecalciferol supplementation (vitD group) from 4.89 ± 0.93 % (mean ± SD) at baseline to 5.68 ±

1.18 % after 4 weeks ($p = 0.018$) to 6.14 ± 1.00 % after 8 weeks ($p < 0.001$) and finally reached 6.35 ± 0.78 % after 12 weeks ($p < 0.001$; Fig. 39 A). There was no significant change in %Tregs during the 12-week study period in the healthy placebo group, and the final mean level was 5.46 ± 0.95 % (vs. 5.35 ± 1.01 % at baseline; Fig. 39 A). Between group comparisons revealed significantly higher percentage of Tregs after 8 and 12 weeks in the healthy vitD group compared to the healthy placebo group (Fig. 39 A). Furthermore, after the cholecalciferol supplementation, the increasing percentage of Tregs in healthy participants correlated significantly with increasing serum 25(OH)D levels from baseline to week 12 in the vitD group, shown by Pearson correlation of delta serum 25(OH)D levels versus delta %Tregs after 12 weeks of supplementation ($p \leq 0.001$, $r = 0.640$) (Fig. 39 E).

In contrast to these results, the mean percentage of Tregs in newly diagnosed T1D patients did not change during the whole study duration, neither in the vitD nor in the placebo group. In detail, mean %Tregs of newly diagnosed T1D patients were comparable at baseline (6.1±1.9 vs 4.8±1.7%, n.s), after 3 months (5.6±0.8 vs 5.1±1.5%), 6 months (5.7±1.4 vs 5.8±1.8%) and 12 months (5.75±1.48% vs 5.38±1.73%, $p=0.187$) between treatment and placebo group (Fig. 39 C).

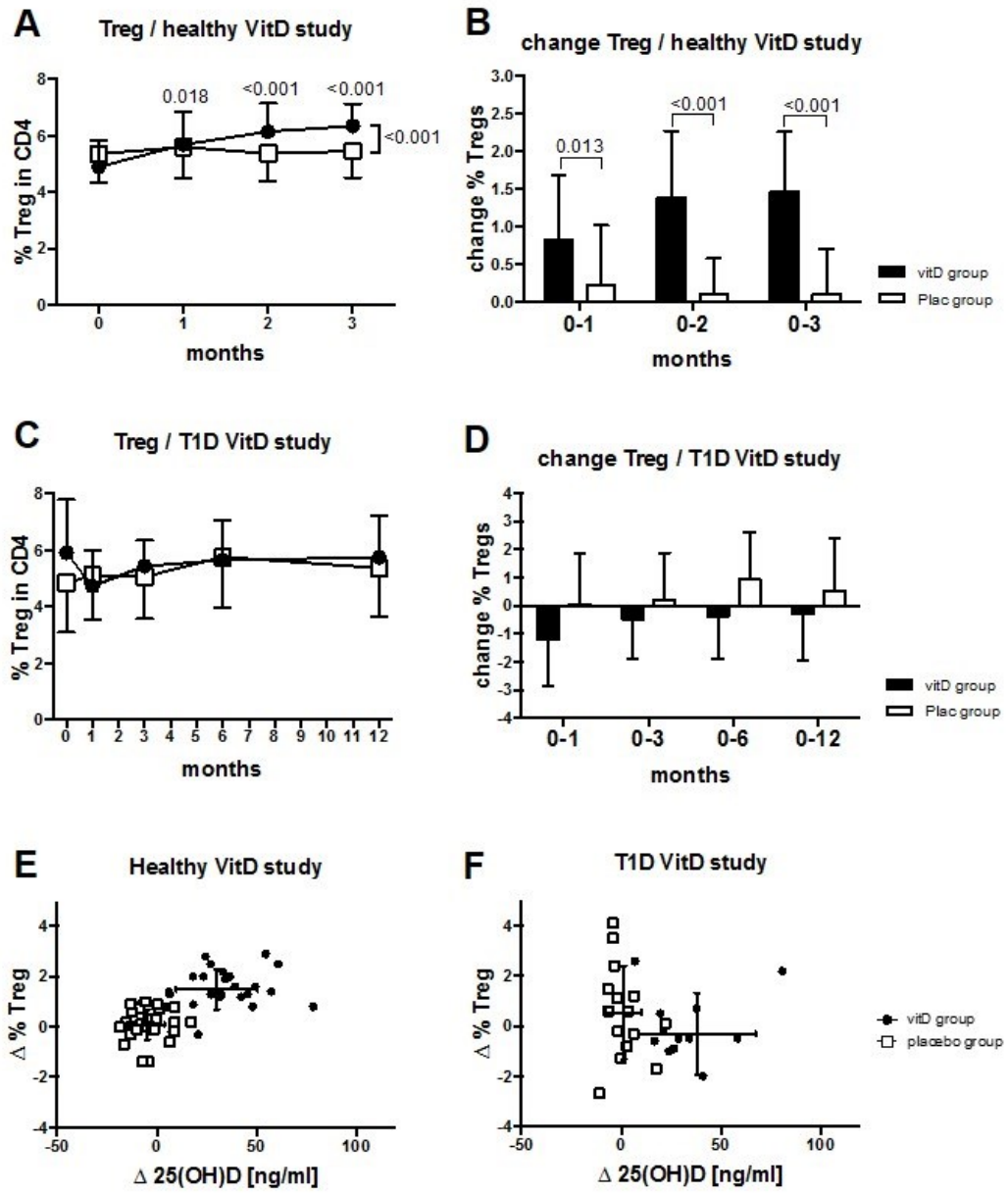

Figure 39: Changes in the % Tregs within $CD4^{pos}$ T cells during oral cholecalciferol supplementation in healthy participants and newly diagnosed T1D patients. **A.** Mean percentage of peripheral Tregs during 12 weeks of cholecalciferol or placebo supplementation in the vitD group (black dots, n=30) and the placebo group (white squares, n=29) in healthy study participants. p-values indicate changes as compared to baseline. **B.** Changes of % Tregs from baseline to 1, 2 and 3 months in healthy participants. **C.** The mean percentage of peripheral Tregs in newly diagnosed T1D patients during a 1-year study period in the vitD group (black dots, n = 14) and the placebo group (white squares, n = 15). **D.** Changes in the % Tregs from baseline to 1, 3, 6 and 12 months in T1D patients. **E.** Pearson correlation of delta serum 25(OH)D levels versus delta %Tregs after 12 weeks of supplementation in healthy controls ($p \leq 0.001$, $r = 0.640$). **F.** Pearson correlation of delta serum 25(OH)D levels versus delta %Tregs after 1 year of supplementation in newly diagnosed T1D patients ($p = 0.028$, $r = -0.407$). Bidirectional bars indicate standard deviation around the mean for the vitD group (black dots) and the placebo group (white squares). Adapted with permission from Clinical Immunology (77).

In healthy participants the most prominent increase in % of peripheral Tregs was found in individuals with a baseline serum vitamin D level in the insufficient range (< 20 ng/ml; Fig. 40). However, increases in the % Tregs due to vitamin D intake were not significant different in individuals with starting serum vitamin D levels below 20 ng/ml when compared to participants with sufficient (20-30 ng/ml) or high (> 30 ng/ml) serum levels.

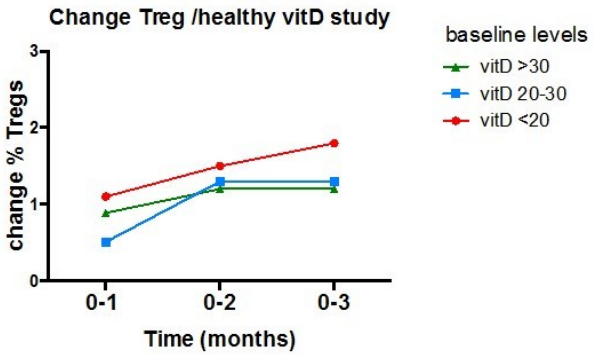

Figure 40: Changes in the % Tregs within $CD4^{pos}$ T cells during oral cholecalciferol supplementation in healthy participants from baseline to month 1, 2 and 3. Mean changes for individuals with insufficient serum vitamin D levels are given in the red line (levels < 20 ng/ml). Data for individuals with a sufficient vitamin D level at baseline are given in blue (levels between 20 to 30 ng/ml) and data for participants with high serum vitamin D levels are given in green (levels > 30 ng/ml).

<u>Increased levels of Tregs normalize soon after termination of cholecalciferol supplementation</u>

Study ID: ADPP003

In healthy participants enrolled in the ADPP003 study the %Tregs within $CD4^{pos}$ T cells increased significantly from 6.2 ± 2.5 % at baseline to 8.1 ± 2.2 % after 8 weeks of treatment and (p=0.001) decreased significantly after the termination of the cholecalciferol supplementation within two weeks to reach a final level of 6.1 ± 1.6 % at the follow up visit after 10 weeks (p=0.002, Fig. 41 A). The expression of the transcription factor Helios in peripheral Tregs showed a slight, not significant trend to decrease upon cholecalciferol treatment (Fig. 41 B).

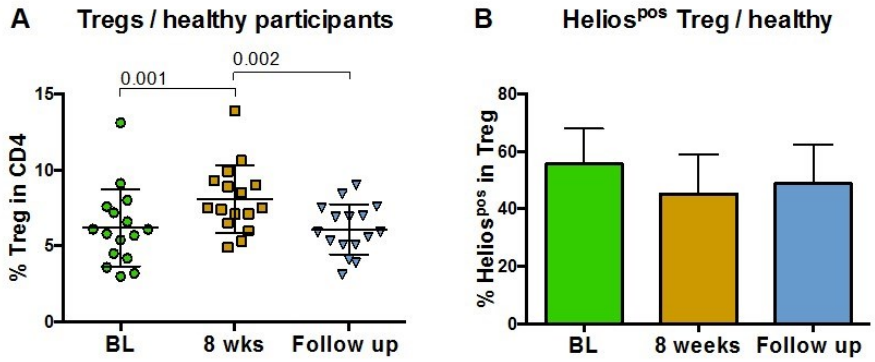

Figure 41: Changes in the % Tregs within $CD4^{pos}$ T cells during and after oral cholecalciferol supplementation in healthy participants. **A.** Mean percentage of peripheral Tregs during 8 weeks of cholecalciferol supplementation and 2 weeks after the last cholecalciferol intake (Follow up). p-values are given on top of the horizontal lines. **B.** No significant changes in the expression of the transcription factor Helios in peripheral Tregs were found during the whole study period, including follow up.

<u>Cholecalciferol treatment improves Treg suppressive capacity in patients with new onset T1D but not in healthy participants</u>

Study IDs: Healthy VitD, ADPP002

Suppressive capacity of Tregs, calculated as the reduction of Teff proliferation in the presence of autologous Tregs, increased in the vitD group of T1D patients participating in the ADPP002 study after 3 months (p=0.049), after 6 months (p<0.001) and after 12 months (p=0.002, Fig. 42 A). In the T1D placebo group, suppressive capacity decreased from baseline to month 12 (p=0.012, Fig. 42 A). After 12 months % suppression in T1D patients was higher in the vitD group than in the placebo group (p=0.017). Changes in the % suppressive capacity (Δ % suppression) from baseline to month 3 were not significantly different between vitD and placebo group (23.5±39.7% vs 14.5±27.3%; p=0.527), while changes from BL to month 6 (33.0±30.8% vs -2.9±34.2%; p=0.009) and month 12 (22.2±47.2% vs -16.6±21.1%; p=0.033) were significantly higher in the vitD group compared to the placebo group (Fig. 42 B)

In healthy individuals, suppressive capacity of Tregs showed a non-significant trend to increase in the vitD group from a mean baseline value of 40 ± 17% to 48 ± 17% after 3 months of cholecalciferol supplementation. In the placebo group a non-significant decrease was shown (Fig. 42 C). However, the changes in the % suppressive capacity (Δ % suppression) from baseline to month 3 were significantly

different between the vitD group and the placebo group (7.6 ± 24% vs -6.6 ± 19%, p=0.033, Fig. 42 D).

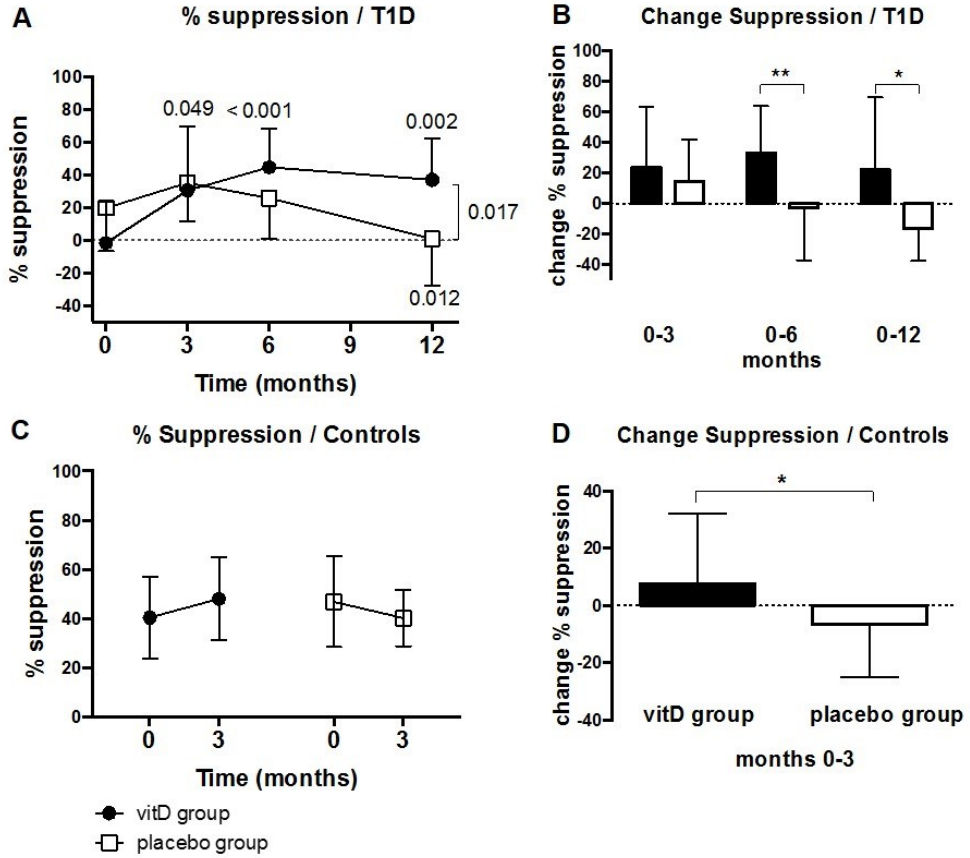

Figure 42: Effects of oral cholecalciferol supplementation on the suppressive potential of FACS sorted Tregs in autologous Treg/Teff co-cultures. **A.** Differences in the suppressive capacity of Tregs from newly diagnosed T1D patients in the vitamin D group (black dots, n=14) and the placebo group (white squares, n=15) during a 1-year study period. **B.** Changes in the % suppression by Tregs from baseline to 3, 6 and 12 months in T1D patients. **C.** Differences in the suppressive capacity of Tregs from healthy participants in the vitamin D group (black dots, n=30) and the placebo group (white squares, n=29) during a 3-months study period. **D.** Change in the % suppression by Tregs from baseline to 3 months in healthy participants. Data given in mean ± SD. p-values are given within the graphs. Adapted with permission from Clinical Immunology (77).

Cholecalciferol treatment does not alter apoptosis of Tregs in healthy participants and patients with new onset T1D

Study IDs: Healthy VitD, ADPP002

Apoptosis of FACS sorted Tregs, assessed within 1 h post-sorting by a standard FACS apoptosis assay, revealed no significant differences between vitD and placebo groups during the whole study period in healthy participants enrolled in the healthy VitD study and newly diagnosed T1D patients participating in the ADPP002 study (Fig. 43). Furthermore, no significant differences were found in changes in % apoptotic cells within Tregs in any of the investigated groups (Fig. 43).

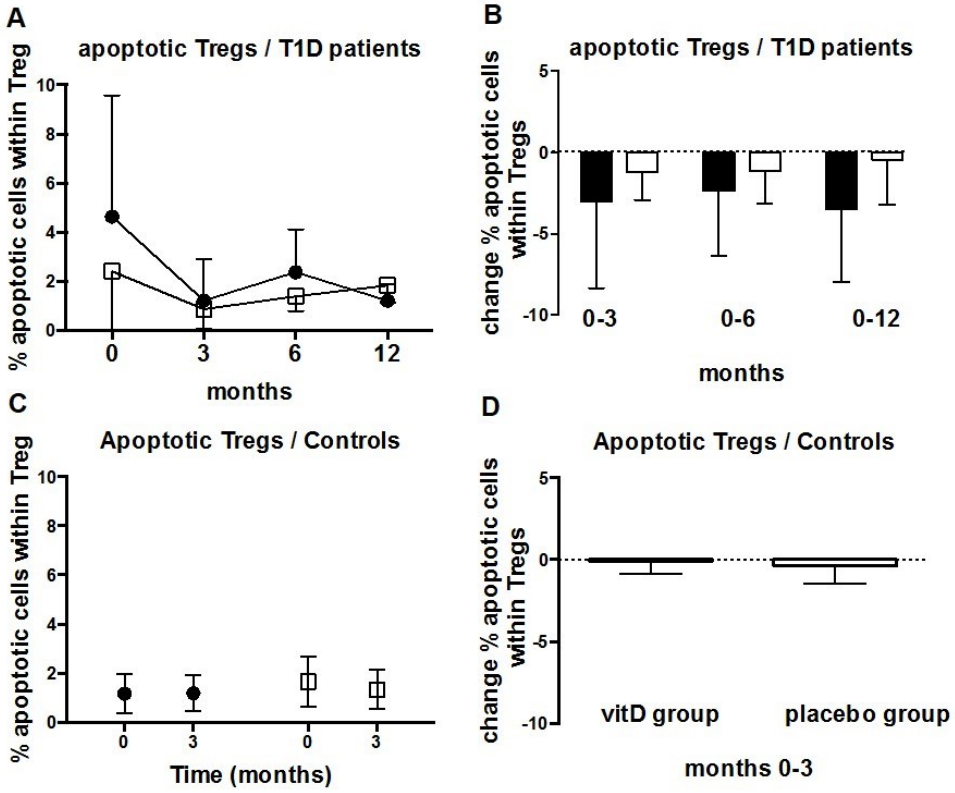

Figure 43: Effects of oral cholecalciferol supplementation on the percentage of apoptotic cells within FACS sorted Tregs. **A.** Differences in the % of apoptotic cells within Tregs from newly diagnosed T1D patients in the vitamin D group (black dots, n=14) and the placebo group (white squares, n=15) during a 1-year study period. **B.** Changes in the % apoptotic cells within Tregs from baseline to 3, 6 and 12 months in T1D patients. **C.** Differences in the % apoptotic cells within Tregs from healthy participants in the vitamin D group (black dots, n=30) and the placebo group (white squares, n=29) during a 3-months study period. **B.** Change in the % apoptotic cells within Tregs from baseline to 3 months in healthy participants. Data given in mean ± SD. Adapted with permission from Clinical Immunology (77).

Effects of cholecalciferol on human peripheral cells of the innate and adaptive immune system

Study IDs: Healthy VitD, ADPP002, ADPP003

In both, healthy participants enrolled in the healthy VitD study and the ADPP003 study and T1D patients, the percentage of T cell subtypes, such as $CD4^{pos}$ and $CD8^{pos}$ memory or naive cells and CD4CD8 double positive cells, did not change significantly nor in the vitD neither in the investigated placebo groups (Fig. 44).

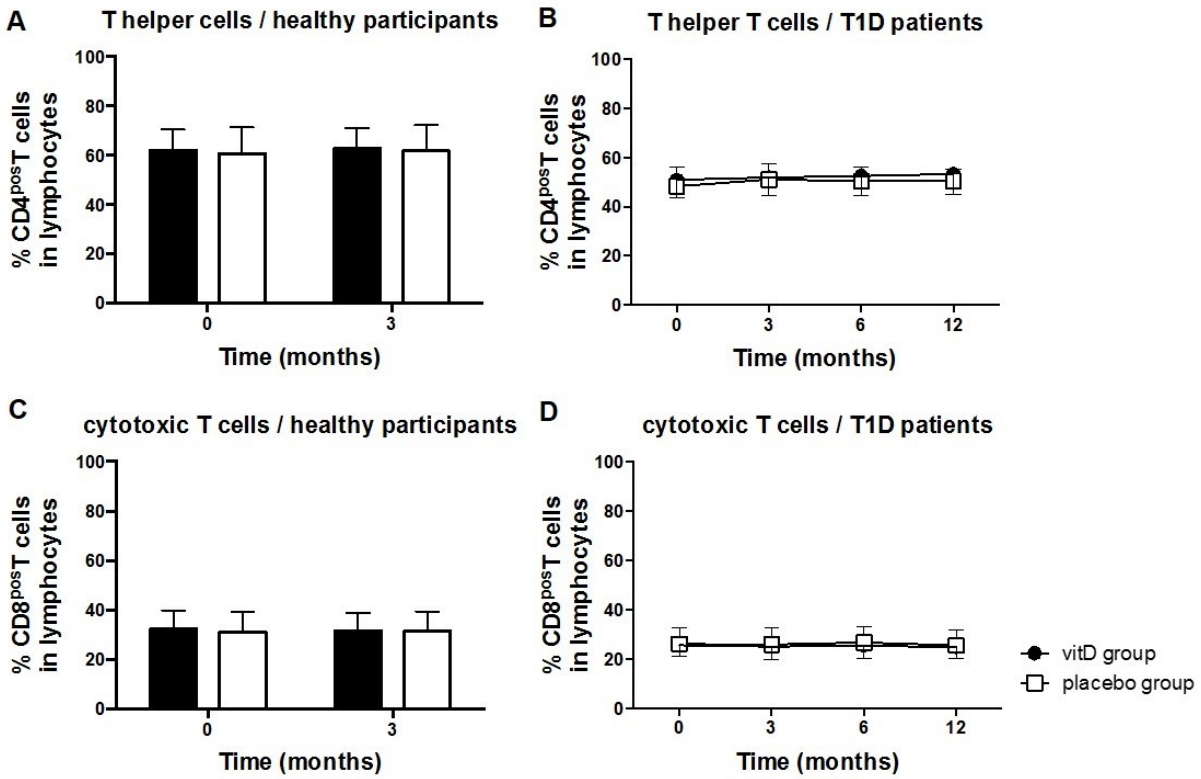

Figure 44: Levels of peripheral $CD4^{pos}$ T helper cells and $CD8^{pos}$ cytotoxic T cells in healthy individuals and newly diagnosed T1D patients participating in two cholecalciferol supplementation trials. **A.** Percentages of $CD4^{pos}$ T helper cells within lymphocytes in healthy participants **B.** Percentages of $CD4^{pos}$ T helper cells within lymphocytes in T1D patients. **C.** Percentages of $CD8^{pos}$ cytotoxic T cells within lymphocytes in healthy participants **D.** Percentages of $CD8^{pos}$ cytotoxic T cells within lymphocytes in T1D patients. Black bars and dots indicate the vitamin D group (n=30 in healthy participants, n=14 in T1D patients), white bars and squares indicate the placebo group (n=29 in healthy participants, n=15 in T1D patients). Data are given in mean ± SD.

Also, cholecalciferol supplementation did not influence the percentage of peripheral B cells, NK cells, NKT cells, myeloid and plasmacytoid dendritic cells, although there was a small decrease in the percentage of myeloid dendritic cells in the healthy participants enrolled in the healthy VitD study compared to the healthy placebo group after 12 weeks (Fig. 45 + 46). But this difference did not remain significant after correcting for multiple comparisons.

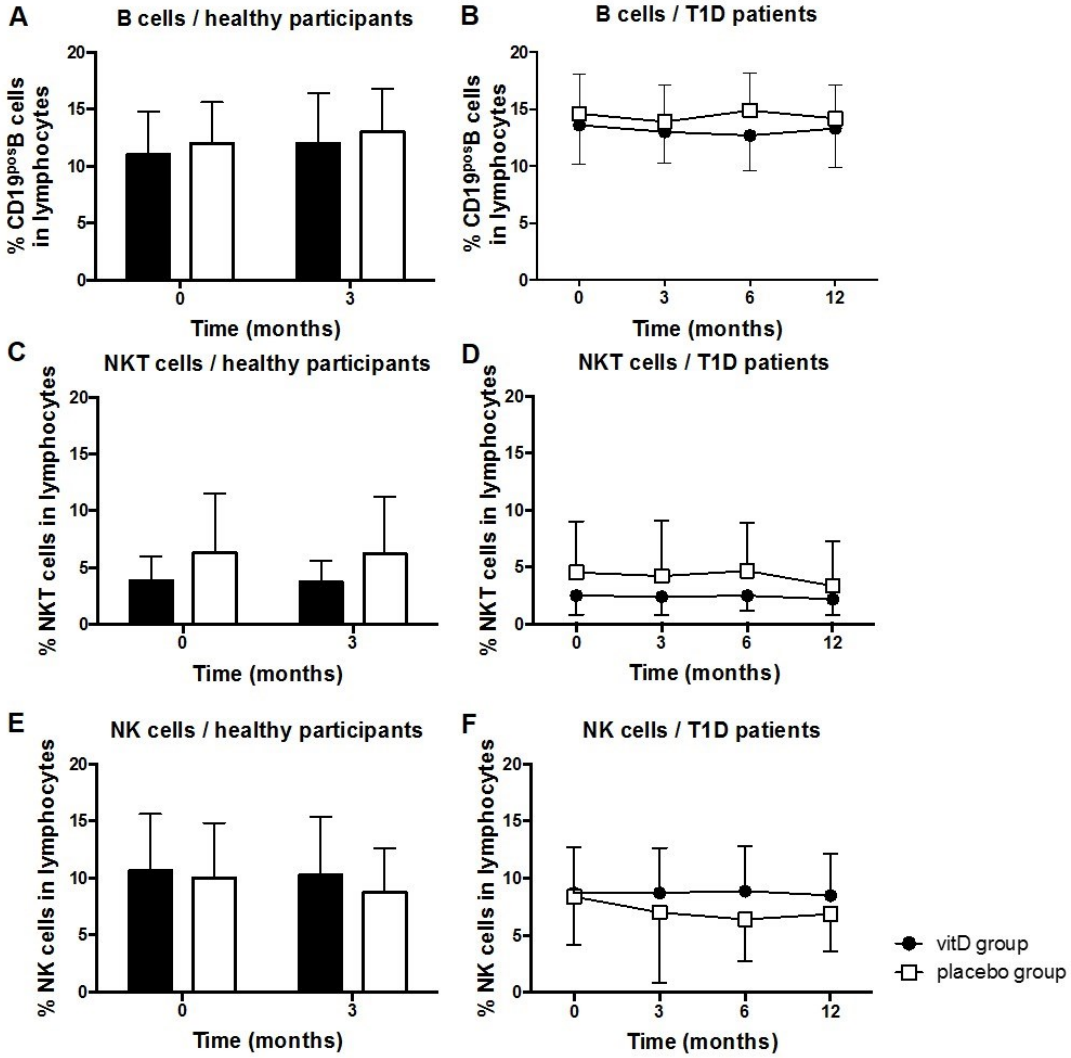

Figure 45: Levels of peripheral B cells, natural killer T-cells (NKT) and natural killer cells (NK) in healthy individuals and newly diagnosed T1D patients participating in two cholecalciferol supplementation trials. **A.** Percentages of $CD19^{pos}$ B cells within lymphocytes in healthy participants **B.** Percentages of $CD19^{pos}$ B cells within lymphocytes in T1D patients. **C.** Percentages of NKT cells within lymphocytes in healthy participants **D.** Percentages of NKT cells within lymphocytes in T1D patients. **E.** Percentages of NK cells within lymphocytes in healthy participants **F.** Percentages of NK cells within lymphocytes in T1D patients. Black bars and dots indicate the vitamin D group (n=30 in healthy participants, n=14 in T1D patients), white bars and squares indicate the placebo group (n=29 in healthy participants, n=15 in T1D patients). Data are given in mean ± SD.

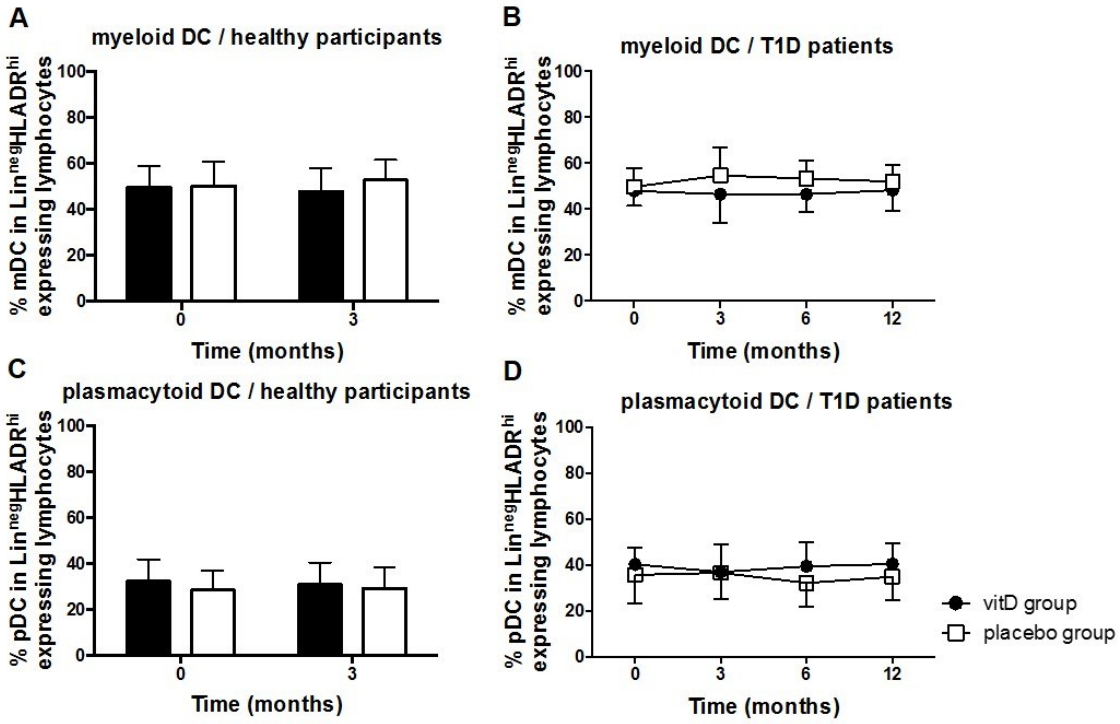

Figure 46: Levels of peripheral myeloid and plasmacytoid dendritic cells (DC) in healthy individuals and newly diagnosed T1D patients participating in two cholecalciferol supplementation trials. **A.** Percentages of myeloid DCs within lymphocytes in healthy participants **B.** Percentages of myeloid DCs within lymphocytes in T1D patients. **C.** Percentages of plasmacytoid DCs within lymphocytes in healthy participants **D.** Percentages of plasmacytoid DCs within lymphocytes in T1D patients. Black bars and dots indicate the vitamin D group (n=30 in healthy participants, n=14 in T1D patients), white bars and squares indicate the placebo group (n=29 in healthy participants, n=15 in T1D patients). Data are given in mean ± SD.

Changes in the absolute numbers of neutrophilic granulocytes and monocytes in the peripheral blood of all study participants revealed no significant changes upon cholecalciferol treatment (data not shown).

Additionally, in healthy individuals from the ADPP003 study and newly diagnosed T1D patients participating in the ADPP002 study $CD4^{pos}$ Th1, Th2 and Th17 cells in the peripheral blood did not significantly change upon cholecalciferol supplementation (Fig. 47).

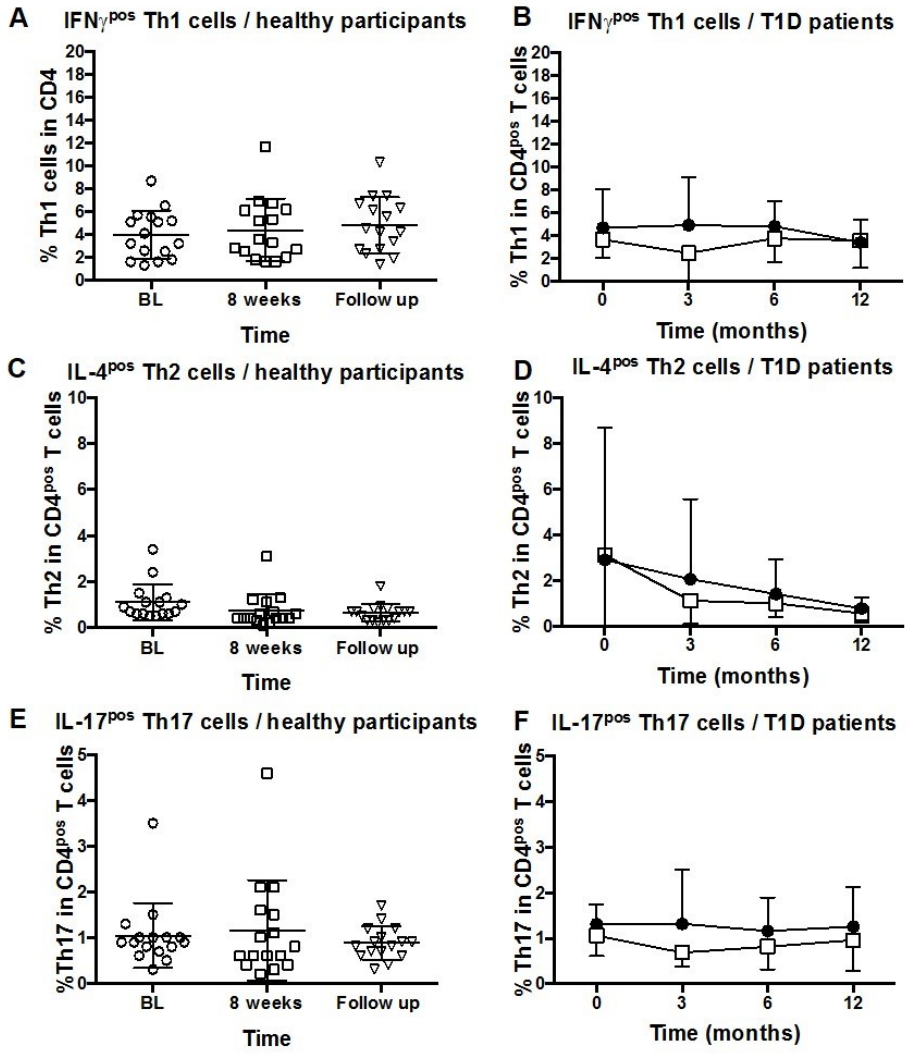

Figure 47: Levels of peripheral Th1, Th2 and Th17 cells in healthy individuals and newly diagnosed T1D patients participating in two cholecalciferol supplementation trials. **A.** Percentages of Th1 cells within $CD4^{pos}$ T cells in healthy participants (n=16) **B.** Percentages of Th1 cells within $CD4^{pos}$ T cells in T1D patients. **C.** Percentages of Th2 cells within $CD4^{pos}$ T cells in healthy participants (n=16) **D.** Percentages of Th2 cells within $CD4^{pos}$ T cells in T1D patients. **E.** Percentages of Th17 cells within $CD4^{pos}$ T cells in healthy participants (n=16) **F.** Percentages of Th17 cells within $CD4^{pos}$ T cells in T1D patients. In the graphs with data from newly diagnosed T1D patients, black dots indicate the vitamin D group (n=14), white squares indicate the placebo group (n=15). Data are given in mean ± SD.

Cholecalciferol affects immune cells associated to the lamina propria

Study ID: ADPP003

Cholecalciferol supplementation of young healthy participants (demographics shown in table 8) did not change the percentage of T cells ($CD3^{pos}$ cells) within lymphocytes but it caused significant changes in subtypes of T cells. In fact, a significant increase in the percentage of $CD4^{pos}$ T helper cells in all T cells ($CD3^{pos}$) isolated from biopsies from the gastric antrum region was shown. The median baseline-value of 12.48% (IQR: 9.05-21.78%) increased to 16.46% (13.36-25.27%) after 8 weeks (p = 0.025, Fig. 48 A). $CD8^{pos}$ cytotoxic T cells within $CD3^{pos}$ T cells increased significantly in the gastric antrum region from a median baseline value of 30.25% (IQR: 22.64-43.33%) to 42.11% (IQR: 25.97-49.94%, p=0.037), in the appendix region from baseline 12.69% (IQR 11.49-16.67%) to 18.56% (IQR 12.17-24.46%, p = 0.005, Fig. 48 B) and in the right colon region from baseline 12.75% (IQR 9.34-18.27%) to 15.09% (IQR 12.45-19.96%, p = 0.035, Fig. 48 B). These changes were not reflected in the ratio CD4/CD8 as this didn´t change significantly upon cholecalciferol intake (Fig. 48 C). The % of Treg within $CD4^{pos}$ T cells also remained unchanged upon cholecalciferol treatment for 8 weeks in all investigated regions of the human intestine, as shown in Fig. 48 D. The expression of the Helios marker in Tregs decreased especially in samples taken from the upper region of the intestine and this decrease reached significance in the Ileum (43.09% [22.68-55.55%] vs 20.44% [16.58-27.79%]; p=0.03, Fig. 48 E).

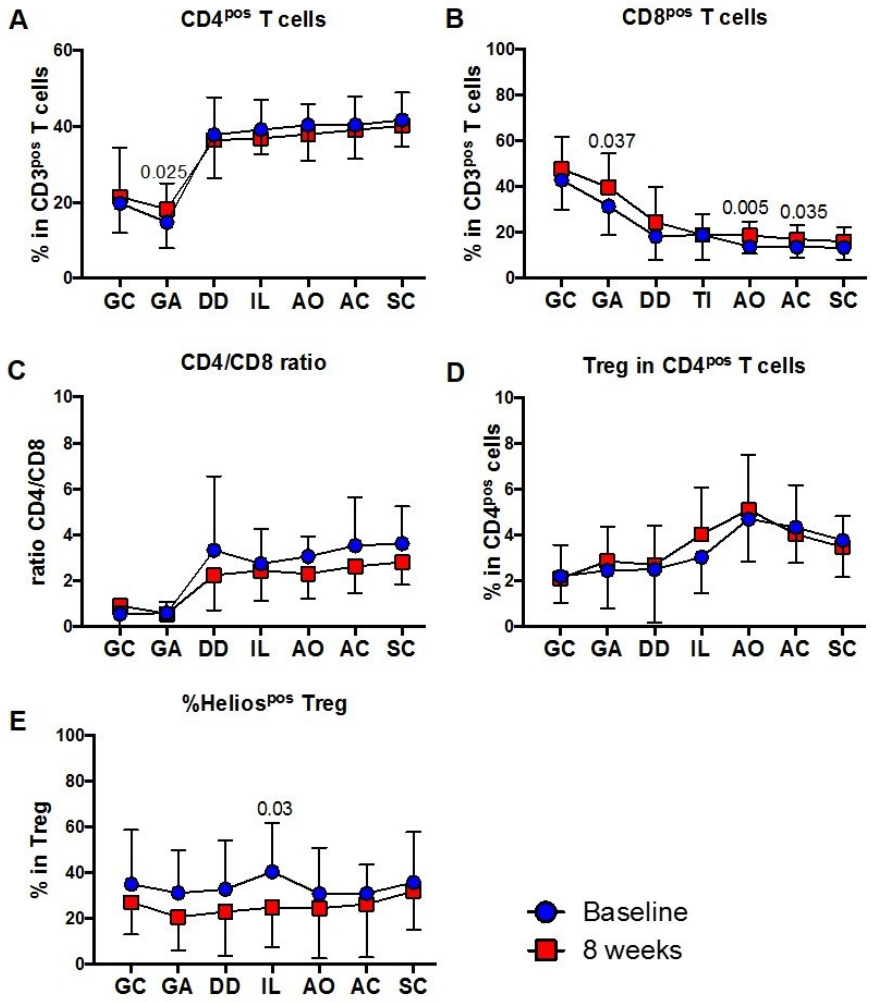

Figure 48: Changes in the percentages of T cell subtypes after 8 weeks of oral cholecalciferol supplementation in the investigated regions of the gastro-intestinal mucosa. **A.** Significant increased levels of $CD4^{pos}$ T helper cells in the gastric antrum region **B.** Significant changes in the % $CD8^{pos}$ cytotoxic T cells in gastric antrum, the appendiceal orifice and the ascending colon region **C.** No significant changes in the CD4/CD8 ratio **D.** no significant changes in the % Tregs **E.** Significant increased levels of $Helios^{pos}$ Tregs in the terminal ileum region. GC: gastric corpus region, GA: gastric antrum region, DD: duodenum, TI: terminal ileum region, AOR: appendiceal orifice region, AC: ascending colon region, SC: sigmoid colon region.

Effects of liraglutide treatment on human immune cells *in vitro* and *in vivo*

Liraglutide treatment increases the proliferation of human PBMCs *in vitro*

Study ID: ADPP001 subgroup analysis

A 96 hour exposure of freshly isolated PBMCs from healthy humans to different concentrations of liraglutide (trade name Victoza®, Novo Nordisk, Denmark) led to an increased proliferation in a dose-dependent manner (Fig. 49 A). Proliferation of PBMCs stimulated with anti CD3/CD28 beads did not change after 96 h of exposure to liraglutide compared to matrix (Fig. 49 B).

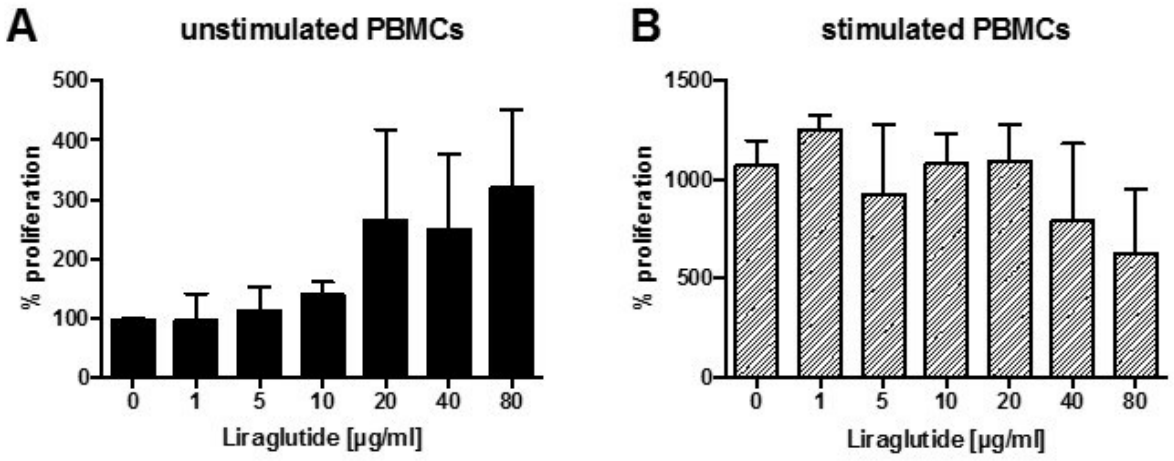

Figure 49: Effect of 96h *in vitro* exposure of human healthy PBMCs to the GLP1 analogue liraglutide. **A** Increased cell-proliferation after addition of liraglutide to unstimulated PBMCs from 3 healthy donors (black bars). **B** Changes in proliferation of stimulated PBMCs after addition of liraglutide (dotted bars, n=3). Stimulation was induced with anti-CD3/CD28 beads (1bead/cell). Reference value (100% proliferation) is shown in Fig. 49 A (0 µg/ml liraglutide). All measurements were done in triplicates. Data given as mean ± SD.

Liraglutide treatment does not increase apoptosis of PBMCs *in vitro*

Study ID: ADPP001 subgroup analysis

Exposure to 10 µg/ml liraglutide, which is in accordance with the approximately 100 time higher concentration than the plasma concentration in patients after the injection of 1.4 mg Victoza, for a 96h cell culture period did not induce enhanced apoptosis in PBMCs from healthy donors compared to exposure to matrix. In detail no significant

difference was found in the percentages of apoptotic cells in PBMCs treated with matrix (1.2 ± 0.1%) and PBMCs treated with 10 µg/ml liraglutide (1.0 ± 0.1%; Fig. 50).

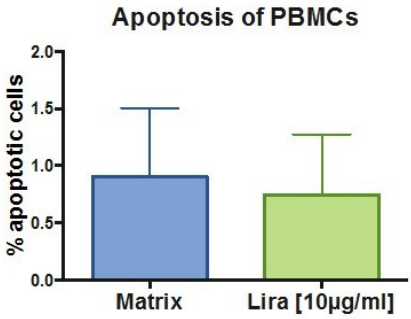

Figure 50: No effect of 96h liraglutide exposure on apoptosis of human healthy PBMCs. All measurements were done in triplicates with PBMCs from 3 healthy donors. Data given as mean ± SD.

<u>Liraglutide treatment does not increase production of IFN-γ, IL-4 or IL-17 in Th cells *in vitro*</u>

Study ID: ADPP001 subgroup analysis

Liraglutide exposure [10 µg/ml] of PBMCs from healthy humans in primary cell cultures for 96h did not influence the percentage of IFN-γ producing Th1-, IL-4 producing Th2- or IL-17 producing Th17-cells within $CD4^{pos}$ T cells when compared to matrix treatment (Fig. 51).

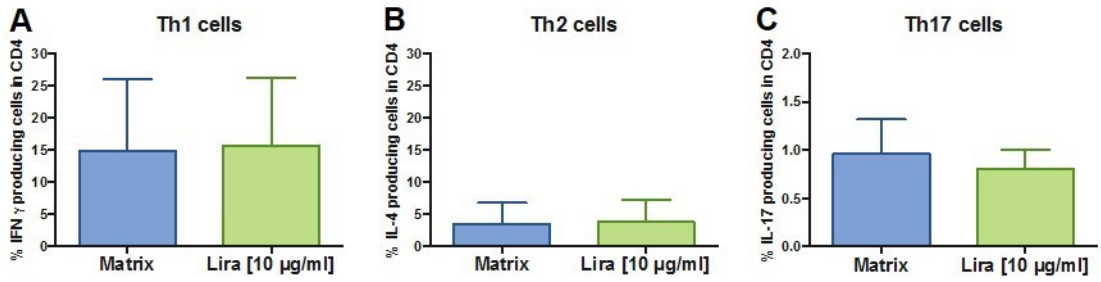

Figure 51: No effect of 96h liraglutide exposure on the % Th1, % Th2 and % Th17 cells within $CD4^{pos}$ T helper cells from healthy donors. **A.** Percentage of IFN-γ producing Th1 cells within $CD4^{pos}$ T cells treated with matrix (blue bar) or liraglutide (green bar). **B.** Percentage of IL-4 producing Th2 cells within $CD4^{pos}$ T cells treated with matrix (blue bar) or liraglutide (green bar). **C.** Percentage of IL-17 producing Th17 cells within $CD4^{pos}$ T cells treated with matrix (blue bar) or liraglutide (green bar). All measurements were done in triplicates with PBMCs from at least 3 healthy donors. Data given as mean ± SD.

Liraglutide treatment induces a trend towards increased suppressive capacity of Tregs *in vitro*

Study ID: ADPP001 subgroup analysis

Suppressive capacity of Tregs from healthy humans, calculated as the reduction of Teff proliferation in a Treg:Teff co-culture (ratio 1 Treg : 4 Teff), increased upon a 96 h exposure to 10 µg/ml liraglutide (29 ± 8%) when compared to matrix treatment (18 ± 9%). This trend towards increased suppression after Liraglutide treatment was not significant (p = 0.071; Fig. 52).

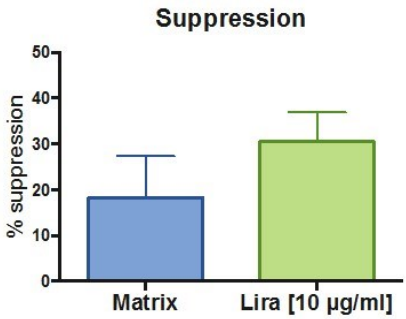

Figure 52: Effect of 96h *in vitro* liraglutide exposure on the suppressive potential of FACS sorted Tregs in autologous Treg/Teff co-cultures (Ratio 1 Treg: 4 Teff). A trend towards increased suppressive capacity (p=0.071) appears due to 96h exposure to liraglutide [10µg/ml] when compared to matrix-treatment [0µg/ml]. All measurements were done in triplicates with PBMCs from at least 3 healthy donors. Data given as mean ± SD.

Treatment with liraglutide but not saxagliptin significantly changes the percentage of $CD4^{pos}$ Tregs in the peripheral blood of healthy participants

Study ID: ADPP004

In total 15 healthy adults were included in this randomized trial. All participants were randomly chosen for liraglutide (Victoza®, Lira group) or for the saxagliptin (Onglyza®, Saxa group) treatment. Because of the different application mode of the two investigated treatments (liraglutide as daily self-injection versus DPP-4 inhibitor as daily oral intake) it was not possible to conduct this study in a double-blinded way. Nevertheless the whole laboratory staff was blinded for the group allocation of the participants. Liraglutide was initially applied in a starting dose (0.6 mg/day) for 1 week followed by self-injection of 1.2 mg/day for 3 weeks. Saxagliptin was taken

orally in a constant dose (5 mg/day) for the whole duration of 4 weeks. In total 4 visits were conducted at baseline, after 2, after 4 and after 8 weeks (follow up visit).
Baseline demographics for all participants are shown in table 12. All 15 participants completed all visits, but as shown in the adapted consort flow diagram (Fig. 10) one participant in the liraglutide group was excluded from analysis (all visits) because of false information about smoking and infection status. Finally, data from 6 participants in the liraglutide group and from 8 participants in the saxagliptin group were analyzed.

ADPP004 Baseline	lira group	saxa group	p value
n	6	8	
Gender (% female)	67%	63%	
BMI (kg/m^2)	24.81 ± 4.24	24.17 ± 4.88	0.295
Age (years)	26.5 (23.5 - 31)	22.5 (20 - 29)	0.741
NLR	2.03 ± 1.30	1.44 ± 0.58	0.652
CD4/CD8 ratio	2.5 (1.9-3.2)	2.5 (1.5-3.4)	0.181
Tregs in CD4 (%)	3.4 (3.3-3.8)	4.1 (3.6-4.9)	0.076

Table 12: Demographic data of our studied populations at time of recruitment. Data are presented as mean ± SD when normally distributed or median + (IQR) when not normally distributed. NLR = neutrophile to lymphocyte ratio.

The percentage of regulatory T cells (%Treg) within CD4^{pos} T cells increased significantly from a median baseline level of 3.4% (IQR: 3.3-3.8%) to 4.1% (IQR: 3.6-4.9%) after 4 weeks of subcutaneous self-injection of liraglutide (0.6 mg/ day in the first week, followed by 1.2 mg/day for 3 weeks). Wilcoxon analysis showed a significant increase of %Treg resulting in a p-value of 0.031 (Fig. 53 and table 13). In contrast to this, oral intake of DPP-4 inhibitors (Saxagliptin) for 4 weeks, did not result in significant changes of %Tregs (p = 0.875). After termination of liraglutide administration, the elevated levels of Tregs were stable until the follow-up visit after 8 weeks (Fig. 58).
No significant changes were found in the percentage of Tregs expressing the transcription factor Helios in any of the investigated groups during the whole study period, although there was a trend towards elevated Helios expression during the administration of liraglutide followed by a decreased expression of Helios after termination of liraglutide administration in the lira group (Fig. 58; median values: BL: 42% (35-60%), 4 weeks: 58% (55-59%), 8 weeks: 49% (23-61%)).

The percentage of Tregs within $CD4^{pos}$ T cells at baseline was higher in the saxa group compared to the lira group, but this difference was not significant (table 12). In contrast, the percentage of $Helios^{pos}$ cells within Tregs was significantly higher in the saxa group compared to the lira group at baseline (68% (57-71%) vs 42% (35-60%), p = 0.043) but not after 8 weeks (p = 0.107, Fig. 53).

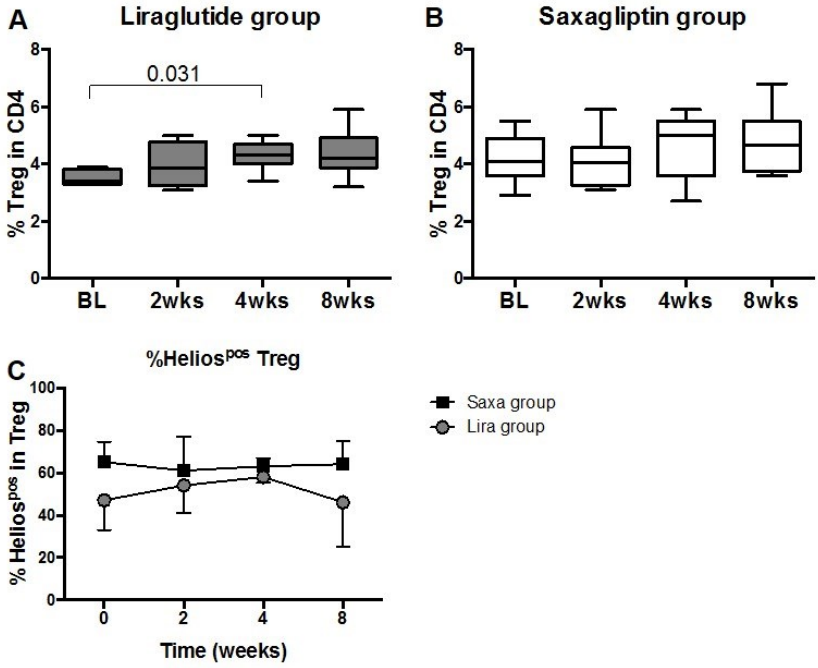

Figure 53: Changes in % Tregs and expression of the transcription factor Helios upon *in vivo* liraglutide- or saxagliptin treatment of healthy participants. **A.** Changes in % Tregs within $CD4^{pos}$ T cells in the liraglutide treated group (n=6). Liraglutide was initially applied in a starting dose (0.6 mg/day) for 1 week followed by self-injection of 1.2 mg/day for 3 weeks. After additional 4 weeks a follow up visit (week 8) was performed. **B.** Changes in % Tregs within $CD4^{pos}$ T cells in the saxagliptin treated group (n=8). Saxagliptin was taken orally in a constant dose (5 mg/day) for the whole duration of 4 weeks. After additional 4 weeks a follow up visit (week 8) was performed. **C.** Changes in the % Heliospos cells within Tregs in the liraglutide treated group and the saxagliptin treated group. The medication was given for 4 weeks and a follow up visit was performed at week 8. Normally distributed data are given in mean ± SD, not normally distributed data are given in boxplots with median + IQR, whiskers indicate the minimum and maximum values.

No effects of liraglutide or saxagliptin administration on human peripheral cells of the innate and adaptive immune system

Study ID: ADPP004

In both investigated groups, treated with liraglutide (lira group) or saxagliptin (saxa group), the percentage of T cell subtypes, such as $CD4^{pos}$ and $CD8^{pos}$ cells or Th1, Th2 and Th17 cells did not change significantly. Also, liraglutide or saxagliptin administration did not influence the percentage of peripheral NK cells, NKT and iNKT cells, myeloid and plasmacytoid dendritic cells (table 13).

	Liraglutide			**Saxagliptin**		
Cell type	**Baseline**	**4 weeks**	**p-value**	**Baseline**	**4 weeks**	**p-value**
CD4 (% of lympho)	54 (45-56)	56 (40-57)	0.313	48 (36-55)	47 (35-52)	0.297
CD8 (% of lympho)	21 (18-29)	22 (16-36)	0.625	26 (24-29)	28 (24-31)	0.219
Treg (% of CD4)	3.4 (3.3-3.8)	4.3 (4.0-4.7)	**0.031**	4.1 (3.6-4.9)	5.0 (3.6-5.5)	0.875
Helios (% of Treg)	42 (35-60)	58 (55-59)	0.156	68 (57-71)	62 (60-67)	0.688
Th1 (% of CD4)	5.7 (2.6-8.0)	6.7 (4.0-11)	0.563	5.4 (2.6-8.3)	5.9 (3.9-9.4)	0.578
Th2 (% of CD4)	0.55 (0.10-1.20)	0.85 (0.4-1.5)	0.531	1.3 (0.43-2.0)	0.70 (0.60-2.9)	0.578
Th17 (% of CD4)	0.60 (0.35-1.0)	0.65 (0.5-0.98)	0.375	1.1 (0.28-3.1)	1.0 (0.60-4.50)	0.313
NK (% of lympho)	8.2 (7.7-11)	6.9 (6.1-13)	0.313	11.0 (2.4-15.0)	9.6 (4.6-18.0)	0.936
NKT (% of lympho)	3.8 (2.6-6.3)	3.4 (2.5-8.9)	0.999	3.6 (2.0-5.4)	2.9 (2.9-4.4)	0.468
iNKT (% of lympho)	0.13 (0.01-0.21)	0.20 (0.06-0.26)	0.375	0.07 (0.02-0.16)	0.03 (0.01-0.16)	0.999
pDC (% of lympho)	28 (17-38)	34 (20-37)	0.813	28 (18-40)	30 (22-42)	0.813
mDC (% of lympho)	57 (47-65)	59 (53-69)	0.999	52 (38-62)	49 (44-67)	0.375

Table 13: Overview for the changes in the % of peripheral immune cell subtypes after a 4 week liraglutide or saxagliptin intervention. Data are given in median + IQR. Wilcoxon-test was used for the calculation of significant changes.

Discussion

The primary aim of this thesis was to investigate the role of peripheral human immune cells involved in either immune regulation or in autoimmune responses in the context of type 1 diabetes mellitus. To face this scientific questions multiple clinical and *in-vitro* studies were performed. Firstly peripheral immune cells from participants of a big clinical cohort study were investigated, aiming to describe differences in the marker expression or functionality of the cells from unrelated healthy participants, T1D patients and related first-degree relatives of the patients involved in the study. Secondly, a randomized, placebo controlled double-blinded trial with a 12 weeks cholecalciferol supplementation of healthy participants was performed to investigate the impact of a high-dose cholecalciferol supplementation on human peripheral immune cells and to gain information about the safety of this treatment. Thirdly, a randomized, placebo controlled double-blinded trial with a 12 months cholecalciferol supplementation of newly diagnosed T1D patients was performed to investigate the role of cholecalciferol in the immune regulation in the pathophysiology of T1D. Fourthly, a pilot trial with a 8 weeks high-dose cholecalciferol supplementation and the sampling of gut biopsies of healthy adults was performed to investigate the effect of cholecalciferol supplementation on the gastrointestinal immune system. Fifthly, the expression of the receptor for glucagon-like peptide 1 (GLP-1) on different peripheral immune cell subtypes was investigated *in vitro* using samples from healthy controls and T1D patients. Sixthly, a randomized clinical trial with healthy participants was performed comparing the effect of a 4 weeks liraglutide or saxagliptin treatment, which are both incretin based medications, on peripheral human immune cells.

T1D is an autoimmune disease caused by destruction of insulin producing β-cells by self-reactive immune cells. It has been shown that T1D is a T cell dependent autoimmune disease and that regulatory T cells (Tregs) play a major role in the failure process of immune regulation. First reports about reduced numbers of Tregs in T1D patients (19) have been disproved by studies that show that the % Tregs in T1D patients is similar to that of healthy controls (20–22). Discrepancies could come from heterogeneity within the Tregs compartment and the lack of an agreement about the exact marker definition for the quantification of Tregs. In our cohort study, investigating peripheral immune cells from 179 T1D patients, 58 siblings and children of the patients and 136 healthy, unrelated volunteers the percentage of Tregs,

defined by the staining of the most common and well established marker combination, was also similar in all groups. However, we found a significant decrease in the suppressive capacity of Tregs from T1D patients when compared to healthy unrelated controls. This functional defect of Tregs from T1D patients was already described in smaller cohort studies before, using more unspecific markers for Treg identification (21,78). Furthermore, apoptosis was significantly increased in Tregs from our T1D patients when compared to healthy controls, which confirms former findings about increased apoptosis of $CD4^{pos}CD25^{hi}$ cells from recent-onset T1D patients when compared to healthy controls or longstanding T1D patients (79). We also found a trend towards increased apoptosis of effector T cells in samples from T1D patients when compared to healthy controls, which reached significance in the subgroup of newly diagnosed T1D patients when compared to age-adjusted healthy controls. All this differences where not correlated to age or metabolic parameters but the differences found in the subgroups of T cells, namely $CD4^{pos}$ and $CD8^{pos}$ T cells with naive or memory phenotype respectively, were strongly correlated to age when healthy samples were compared to samples from T1D patients. Therefor these parameters were not taken into account for the clinical trials performed with vitamin D or incretin-based drugs. Additionally, we found a significant increase in the percentage of $CD19^{pos}$ B cells in the peripheral blood of T1D patients when compared to healthy controls, which is a confirmation of the common signs for autoimmunity, but which has also raised interest recently because of the positive results from animal models and clinical studies using a B cell depleting agent (reviewed by Hinman et al. (80)).

Cellular therapies using Tregs have been suggested for the treatment of autoimmune diseases, including T1D, transplantation and graft versus host diseases (36,39,81). Defects in immune regulation associated with T1D could arise from dysfunctional Tregs, as confirmed in our cohort study, but also from enhanced resistance of effector T cells to regulatory signals (26,27). In animal models, a high number of Tregs has already been shown to decrease the rejection of transplanted organs and to reduce the progression of autoimmune diseases (82). In children suffering from T1D adoptive transfer of *in vitro* expanded Tregs was reported to significantly increase the number of peripheral Tregs and to have positive outcomes concerning β-cell function, but the long time safety of this therapy remains unclear (81). Immunosuppressive compounds, such as rapamycin alone or in combination with low

doses of IL-2, selectively expands functional Tregs from T1D patients but this is always accompanied by severe safety concerns (83–85). In animal studies it has already been demonstrated that a vitamin D treatment can be associated with an increased number and a modified function of Tregs as well as a shift towards anti-inflammatory signaling (86–89). Recently, Takiishi et al. reported that a life-long diet with high doses of cholecalciferol increases % Tregs and decreases effector T cells in NOD (non obese diabetic) mice (49). Our randomized, placebo controlled and double-blinded cholecalciferol supplementation studies are first to show a significant influence of a high-dose cholecalciferol supplementation on the percentage of peripheral Tregs in healthy humans and a significant increase in functional capacity of Tregs in newly diagnosed T1D patients. In all three studies, serum 25(OH)D increased to a level considered to reduce the incidence rates of T1D, multiple sclerosis and rheumatoid arthritis (90–93). Large epidemiological studies have confirmed a links between insufficient 25(OH)D levels and a wide range of human autoimmune disorders, cardiovascular diseases and cancer (94). Toss et al. reported that a daily supplementation of 40 µg cholecalciferol (1600 IU) is sufficient to obtain serum 25(OH)D levels above 50 nmol/l (20 ng/ml) in elderly people which is considered to be very safe and sufficient for beneficial effects on bone and mineral metabolism (95). Since the immunomodulatory effects of vitamin D are dependent on high local concentrations of the bioactive form $1,25(OH)_2D_3$ (calcitriol) we decided to supplement the inactive form of vitamin D (cholecalciferol) at high doses (70IU/kg/day). Cholecalciferol serves as substrate for liver enzymes (CYP2R1 and CYP27A1) that catalyze the conversion into the main storage form 25(OH)D. We were able to achieve high local concentrations of 25(OH)D that are necessary for immunomodulatory effects by 1α(OH)ase induction directly in immune cells without any clinical side effects and at the same time avoiding any limiting factors such as the short half-life of calcitriol (4-6 h).

Calcitriol has been shown to induce Tregs by modulating the maturation and tolerogenic properties of antigen presenting cells *in vitro* (96). In cell cultures without the presence of antigen presenting cells, calcitriol directly decreases the proliferation of Tregs, but it preserves the suppressive function and increases the IL-10 production (97). Furthermore, treatment of naive human T cells with calcitriol and IL-2 *in vitro* directly suppresses the production of proinflammatory cytokines while promoting high expression of FoxP3, CTLA-4 and IL-10 in cells with a regulatory

phenotype (98). In our two cholecalciferol intervention trials with healthy participants an increase in serum 25(OH)D levels led to a significant increase of % Tregs in the vitD groups, whereas the levels of peripheral Tregs were stable in the placebo groups. In one of these trials we also performed a follow up visit, conducted 2 weeks after the termination of cholecalciferol supplementation. There we found a significant decrease of the % of Tregs within 2 weeks after the last cholecalciferol intake, re-establing the initial level of Tregs in the peripheral blood. In one of the trials changes in the suppressive function of Tregs after cholecalciferol supplementation were investigated, resulting in a positive trend towards increased function in the vitD group when compared to changes of function in the placebo group. In patients with new onset T1D, on the contrary, cholecalciferol treatment was able to improve suppressive capacity of Tregs significantly, but had no influence on the percentage of Tregs in the peripheral blood. In the placebo group we found a decline in suppressive capacity of Tregs within the first year after T1D diagnosis. This is in line with a report from Hughson et al. (99), who described either a transient loss of suppression within the first 3-6 months or a stable loss of Treg function throughout the first 12 months after T1D onset. An increase in the percentage of Tregs after a cholecalciferol supplementation has been recently reported in newly diagnosed T1D patients although the percentage of Tregs did not differ between treatment and placebo group after 12 months of supplementation (100). In this study the functional capacity of Tregs was not measured but stimulated C-peptide was enhanced in the first 12 months and had less decline until 18 months in the cholecalciferol group. We also report a trend towards a slower decline in fasting C-peptide in newly diagnosed T1D patients who received high doses of cholecalciferol for one year, although this study was not powered to investigate C-peptide changes. Furthermore, a lower daily insulin dose requirement per kg bodyweight after 12 months of supplementation was observed in our vitD group. Safety parameter measurements throughout all our trials are in line with previous studies that have reported no clinically relevant side effects such as hypercalcemia or hypercalciuria after a high-dose cholecalciferol supplementation in patients with relapsing remitting multiple sclerosis (58).

Since all our studies have primary aimed to investigate peripheral Tregs, it remains unclear whether changes in the percentage or function of Tregs from the peripheral blood cells reflect respective changes in tissues or organs. The oral administration of cholecalciferol may be already beneficial in the gastrointestinal lumen by modulation

of the gut microbiota and consequently regulation of innate and adaptive immune responses. To answer the question whether high-dose oral cholecalciferol supplementation influences the immune cell composition in the gastrointestinal tissue in healthy participants we investigated tissue biopsies from 7 different regions throughout the whole human gastrointestinal tract. At baseline we found significant variations in the percentages of T cell subtypes, including Tregs, along the human gastrointestinal tract (75). In a subpart of this study we could show that the cholecalciferol supplementation for 8 weeks resulted in a strong effect on the human microbiome of the upper GI-tract (101). In particular, a reduction of opportunistic pathogens and an increase in richness of bacterial species was obtained. These effects where accompanied by an increased $CD8^{pos}$ T-cell fraction throughout three gut regions, reaching the highest significance in the duodenum. The percentage of Tregs did not change significantly, but the $\%CD4^{pos}$ T cells was increased in the gastric antrum region after 8 weeks of cholecalciferol supplementation. Moreover, the percentage of Tregs co-expressing the transcription factor Helios was decreased significantly in the ileum after 8 weeks of cholecalciferol intake. Whereas first reports identified Helios as a marker for Tregs that originated from the thymus (102), Helios is now known to enhance the expression of FoxP3 and it was recently shown that FoxP3/Helios double positive Tregs have stronger suppressive capacities than $FoxP3^{pos}Helios^{neg}$ Tregs (103). In contrast to our findings in the gastrointestinal tissue samples, we detected no significant changes in the Helios expression of Tregs in the peripheral blood of the participants. There is a need for more clinical studies to investigate the role of vitamin D in the expression of the transcription factor Helios.

In a special region of the human lower gut, the incretin hormone, glucagon-like peptide-1 (GLP1), is secreted postprandial to regulate glucose homeostasis by the L-cells (61,104,105). It potentiates insulin secretion, inhibits glucagon secretion, offers beneficial effects on pancreatic α- and β-cells and suppresses appetite and food intake. Therefore, targeting GLP1 is a promising therapeutic approach in patients with type 2 diabetes mellitus (T2D). Besides this, GLP1 and its analogs, such as exendin-4 and liraglutide, have been reported to exert cardioprotective and vasodilatory actions (106) and to improve vascular endothelial dysfunction and inflammation (107,108).

In the performed *in vitro* experiments we show that the GLP1-R is present in all investigated human immune cell types with the highest expression in monocytes. The

abundant GLP1R expression in monocytes, which was observed in this work, is in line with the findings of other studies (109,110). Recently, anti-inflammatory actions of human monocyte-derived macrophages after GLP1-R agonist treatment were reported (111). This supports the assumption that GLP1 has a direct influence on monocytes (109,112,113). Recruitment of pro-inflammatory macrophages, which are related to monocytes, into the visceral fat is known to be decreased when GLP1 degradation is inhibited in a mouse model of type 2 diabetes (114). Furthermore, our study showed that healthy $CD8^{pos}$ and $CD4^{pos}$ T- cells with a memory phenotype seem to express a higher amount of GLP1-R than those with a naive phenotype. Mature naive T- cells are produced in the thymus and released into the bloodstream and can differentiate into memory T- cells after the recognition of an antigen in case of infection. The majority of these memory T- cells are removed by apoptosis after the infection has been cleared. Only a small proportion of memory T-cells survives and is able to respond immediately in case of re-infection (115). The high expression of the GLP1-R in T-cells which have experienced contact with an antigen leads to the assumption that antigen contact may play a role for the expression of the GLP1-R. We also investigated the expression of the GLP1R in immune cells from newly diagnosed and long-term T1D patients. We found significantly different levels in the expression of GLP1-R on naive and memory $CD4^{pos}$ and $CD8^{pos}$ T cells from longstanding T1D patients compared to newly diagnosed patients or healthy participants. Tregs and B cells from long-term T1D patients showed a significant elevated GLP1-R expression when compared to cells from newly diagnosed T1D patients. The role of the increased GLP1-R expression in longstanding T1D remains open and further studies with higher numbers of participants are needed to assess whether such effects may be clinically relevant for T1D patients. GLP1-R agonists are also known to inhibit lymphocyte migration in cell cultures, to increase the circulating number of Tregs and to delay the onset of T1D in animal models (67,71,116). Due to these findings GLP1-R agonists seem to be ideal candidates for new therapeutic approaches in T1D. Therefore we tested the influence of liraglutide exposure on the functional properties of Tregs *in vitro*. We found a trend towards increased suppressive capacity of Tregs, although the assays where only performed with Tregs from healthy participants, which have no functional alteration in their regulatory capacity as seen in T1D patients. These results are in accordance to the observations about the expression of Glp1r mRNA in murine immune cells and the

fact that activation of the GLP1-R leads to cAMP accumulation in mouse models (66). Increased production of cAMP promotes an anti-inflammatory shift by enhancing IL-10 synthesis and suppression of TNF production and is one known regulatory strategy of Tregs (117). We also observed an increased proliferation of PBMCs from healthy humans exposed to liraglutide in vitro. This is supported by the report that lymphocytes from Glp1−/− mice were hypoproliferative in response to mitogenic stimulation, suggesting that GLP1 may control lymphocyte proliferation in mice (66).

In humans, Hogan et al. reported that liraglutide treatment of patients suffering from psoriasis as well as T2D resulted in an improvement in clinical psoriasis activity (68). This was associated with an increased number of iNKT cells in the circulation and a decreased number of these cells directly in the psoriatic lesions. We performed a pilot study investigating the effects of a 4 week period of liraglutide or saxagliptin treatment on the peripheral immune cell subtypes from healthy participants. We found a significant increase in the percentage of Tregs in the liraglutide group and a non-significant trend towards elevated Tregs levels in the saxagliptin group. These findings are in accordance to reports from animal models about increased Treg numbers in NOD mice after treatment with the GLP1-R agonist exendin-4 (67).

In conclusion the *in vitro* and *in vivo* experiments performed within this thesis provide significant new information about the differences in immune cell numbers and functional properties between healthy conditions and T1D and support the finding that sufficient 25(OH)D serum levels influence Tregs number or function without safety concerns. This confirms the potential of vitamin D used as adjunctive therapy targeting immunomodulation in autoimmune mediated diseases. The obtained data about the distribution of the GLP1-R on peripheral immune cells from healthy controls and diabetic patients and about the response of healthy immune cells to liraglutide treatment *in vitro* and *in vivo*, offer new insights in the possible role of the GLP1-R in the development, maintenance and regulation of human peripheral immune answers. These data will probably serve as rationale for future trials, testing the complementary effect of combination therapies, including high doses of vitamin D and GLP1R-agonists in the context of type 1 diabetes and other autoimmune diseases.

References

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T Cells and Immune Tolerance. Cell. 2008;133(5):775–787.
2. Sakaguchi S, Takahashi T, Nishizuka Y. Study on Cellular Events in Postthymectomy Autoimmune Oophoritis in Mice. J Exp Med. 1982;156:1565–1576.
3. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151–1164.
4. Baecher-Allan CC, Hafler DA. Human regulatory T cells and their role in autoimmune disease. Immunol Rev. 2006 Aug;212:203–216.
5. Buckner JH. Mechanisms of impaired regulation T cells in human autoimmune diseases. Nat Immunol. 2010;10(12):849–859.
6. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. FASEB J. 2012 Jun;26(6):2253–76.
7. Tang. The FoxP3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol. 2008 Mar;9(3):239–244.
8. Ochs HD, Ziegler SF, Torgerson TR. FOXP3 acts as a rheostat of the immune response. Immunol Rev. 2005 ;203:156–164.
9. Hori S, Nomura T, Sakaguchi S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science. 2003;299:1057–1061.
10. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol. 2003;4(4):337–342.
11. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003 ;4(4):330–336.
12. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol. 2005 Dec; 6(12):1219–1227.
13. Apostolou I. In Vivo Instruction of Suppressor Commitment in Naive T Cells. J Exp Med. 2004;199(10):1401–1408.
14. Thornton AM, Shevach EM. Suppressor Effector Function of CD4+CD25+ Immunoregulatory T Cells Is Antigen Nonspecific. J Immunol. 2000;164(1):183–190.
15. Dejean AS, Beisner DR, Ch IL, Kerdiles YM, Babour A, Arden KC, et al. Foxo3 controls the magnitude of T cell immune responses by modulating dendritic cell function. Nat Immunol. 2009;10(5):504–513.
16. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 Control over FoxP3+ Regulatory T Cell Function. Science . 2008 Okt;10; 322:271–275.
17. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation.

Proc Natl Acad Sci U S A. 2008;105(29):10113–10118.

18. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol. 2002;3(11):1097–1101.
19. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest. 2002 Jan;109(1):131–140.
20. Putnam AL, Vendrame F, Dotta F, Gottlieb PA. CD4+CD25high regulatory T cells in human autoimmune diabetes. J Autoimmun. 2005;24:55–62.
21. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TIM. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes. 2005 Jan;54(1):92–99.
22. Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, et al. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes. 2007 Mar; 56(3):604–612.
23. Long SA, Cerosaletti K, Bollyky PL, Tatum M, Shilling H, Zhang S, et al. Defects in IL-2R Signaling Contribute to Diminished Regulatory T-Cells of Type 1 Diabetic Subjects. Diabetes. 2010;59(2):407–415.
24. Marwaha AK, Crome SQ, Berg KB, Qin H, Ouyang Q, Xu L, et al. Cutting Edge: Increased IL-17 − Secreting T Cells in Children with New-Onset Type 1 Diabetes. J Immunol. 2010 Oct; 185(7):3814-3818.
25. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol. 2010 Dec;10(12):849–859.
26. Schneider A, Rieck M, Sanda S, Greenbaum C, Buckner JH, Foxp CD, et al. The Effector T Cells of Diabetic Subjects Are Resistant to Regulation via CD4 +FOXP3+ Regulatory T Cells. J Immunol. 2008;181:7350–7355.
27. Lawson JM, Tremble J, Dayan C, Beyan H, Leslie RDG, Peakman M, et al. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin Exp Immunol. 2008 Dec;154(3):353–359.
28. Atkinson MA. The pathogenesis and natural history of type 1 diabetes. Cold Spring Harb Perspect Med. 2012 Nov;2(11).
29. Leslie RDG, Kolb H, Schloot NC, Buzzetti R, Mauricio D, De Leiva A, et al. Diabetes classification: grey zones, sound and smoke: Action LADA 1. Diabetes Metab Res Rev 2008;(24):511–519.
30. Moltchanova E V, Schreier N, Lammi N, Karvonen M. Epidemiology Seasonal variation of diagnosis of Type 1 diabetes mellitus in children worldwide. Diabet Med. 2009;26:673–678.
31. Harjutsalo V, Sjöberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet. 2008 May;371(9626):1777–1782.
32. Harjutsalo V, Sund R, Knip M, Groop PH. Incidence of type 1 diabetes in Finland. JAMA. 2013 Jul; 310(4):427–428.
33. Maahs DM, Rewers M. Editorial: Mortality and renal disease in type 1 diabetes mellitus--progress made, more to be done. J Clin Endocrinol Metab. 2006 Oct; 91(10):3757–3759.

34. Noble JA, Erlich HA. Genetics of Type 1 Diabetes. Cold Spring Harb Perspect Med. 2012;2(1):a007732–a007732.
35. Tooley JE, Waldron-Lynch F, Herold KC. New and future immunomodulatory therapy in type 1 diabetes. Trends Mol Med; 2012 Mar; 18(3):173–181.
36. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev. 2008 Jun;223:371–390.
37. Notley CA, McCann FE, Inglis JJ, Williams RO. Anti-CD3 therapy expands the numbers of CD4+ and CD8+ Treg cells and induces sustained amelioration of collagen-induced arthritis. Arthritis Rheum. 2010 Jan ;62(1):171–178.
38. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. J Exp Med. 2009 Apr 13;206(4):751–760.
39. Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E. Rapamycin Monotherapy in Patients With Type 1. Diabetes. 2008;57(9):2341–2347.
40. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin d and immune function. Nutrients. 2013 Jul;5(7):2502–2521.
41. Heaney RP. Assessing vitamin D status. Curr Opin Clin Nutr Metab Care. 2011 Sep;14(5):440–444.
42. Baeke, Takiishi, Korf, Gysemans M. Vitamin D: modulator of the immune system. Curr Opin Pharmacol. 2010;10:482–496.
43. Adorini L, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. Hum Immunol. American Society for Histocompatibility and Immunogenetics; 2009 May; 70(5):345–352.
44. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. J Immunol. 2009 Nov;183(9):5458–5467.
45. Gregori S, Giarratana N, Smiroldo S, Uskokovic M, Adorini L. A 1alpha,25-dihydroxyvitamin D(3) analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice. Diabetes. 2002 May; 51(5):1367–1374.
46. Morales-Tirado V, Wichlan DG, Leimig TE, Street SEA, Kasow K a, Riberdy JM. 1α,25-dihydroxyvitamin D3 (vitamin D3) catalyzes suppressive activity on human natural regulatory T cells, uniquely modulates cell cycle progression, and augments FOXP3. Clin Immunol. Elsevier Inc.; 2011 Feb; 138(2):212–221.
47. Essen MR Von, Kongsbak M, Schjerling P, Olgaard K, Ødum N, Geisler C, et al. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. Nat Immunol.; 2010 Apr;11(4):344–349.
48. Peelen E, Knippenberg S, Muris A, Thewissen M, Smolders J, Willem J, et al. Effects of vitamin D on the peripheral adaptive immune system : A review. Autoimmun Rev.; 2011;10(12):733–743.

49. Takiishi T, Ding L, Baeke F, Spagnuolo I, Sebastiani G, Laureys J, et al. Dietary Supplementation with high doses of Regular Vitamin D3 Safely Reduces Diabetes Incidence in NOD Mice when given Early and Long-Term. Diabetes. 2014;63(6):2026–2036.
50. Raab J, Giannopoulou EZ, Schneider S, Warncke K, Krasmann M, Winkler C, et al. Prevalence of vitamin D deficiency in pre-type 1 diabetes and its association with disease progression. Diabetologia. 2014 Feb; 57(5):902–908.
51. Chakhtoura M, Azar ST. The role of vitamin d deficiency in the incidence, progression, and complications of type 1 diabetes mellitus. Int J Endocrinol. 2013 Jan; Epub 2013:148673.
52. Hyppönen E. Vitamin D and increasing incidence of type 1 diabetes-evidence for an association? Diabetes, Obes Metab. 2010 Sep; 12(9):737–743.
53. Zipitis CS, Akobeng AK. Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis. Arch Dis Child. 2008 Jun; 93(6):512–517.
54. Eurodiab Substudy 2 Study Group. Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. Diabetologia. 1999 Jan; 42(1):51–54.
55. Walter M, Kaupper T, Adler K, Foersch J, Bonifacio E, Ziegler AG. No effect of the 1alpha, 25-dihydroxyvitamin D3 on beta-cell residual function and insulin requirement in adults with new-onset type 1 diabetes. Diabetes Care. 2010; 33(7):1443–1448.
56. Bizzarri C, Pitocco D, Napoli N, Di Stasio E, Maggi D, Manfrini S, et al. No Protective Effect of Calcitriol on beta-Cell Function in Recent-Onset Type 1 Diabetes. Diabetes Care. 2010;33(9):1962–1963.
57. Li X. Protective effects of 1-α-hydroxyvitamin D3 on residual β-cell function in patients with adult-onset latent autoimmune diabetes (LADA). Diabetes Metab Res Rev. 2009;3(6):411–416.
58. Smolders J, Peelen E, Thewissen M, Cohen Tervaert JW, Menheere P, Hupperts R, et al. Safety and T cell modulating effects of high dose vitamin D3 supplementation in multiple sclerosis. PLoS One. 2010 Jan; 5(12):e15235.
59. Gabbay MAL, Sato MN, Finazzo C, Duarte AJS, Dib SA. Effect of cholecalciferol as adjunctive therapy with insulin on protective immunologic profile and decline of residual β-cell function in new-onset type 1 diabetes mellitus. Arch Pediatr Adolesc Med. 2012 Jul; 166(7):601–607.
60. Prietl B, Pilz S, Wolf M, Tomaschitz A, Obermayer-Pietsch B, Graninger W, et al. Vitamin D supplementation and regulatory T cells in apparently healthy subjects: vitamin D treatment for autoimmune diseases? Isr Med Assoc J. 2010 Mar;12(3):136–139.
61. Drucker DJ. The biology of incretin hormones. Cell Metab. 2006 Mar; 3(3):153–165.
62. Baggio LL, Drucker DJ. Biology of Incretins: GLP-1 and GIP. Gastroenterology. 2007; 132(6):2131–2157.
63. Bradley DP, Kulstad R, Schoeller DA. Exenatide and weight loss. Nutrition. 2010;26(3):243–249.
64. Brubaker L, Drucker DJ. Structure-function of the glucagon receptor family of G protein-coupled receptors: the glucagon, GIP, GLP-1, and GLP-2 receptors. Receptors Channels. 2002 Jan; 8(3-4):179–188.
65. Pyke C, Heller RS, Kirk RK, Orskov C, Reedtz-Runge S, Kaastrup P, et al. GLP-1 receptor

localization in monkey and human tissue; Novel distribution revealed with extensively validated monoclonal antibody. Endocrinology. 2014 Jan; 155(4):1280–1290.

66. Hadjiyanni I, Siminovitch K a., Danska JS, Drucker DJ. Glucagon-like peptide-1 receptor signalling selectively regulates murine lymphocyte proliferation and maintenance of peripheral regulatory T cells. Diabetologia. 2010 Jan 8; 53(4):730–740.
67. Xue S, Wasserfall CH, Parker M, Brusko TM, McGrail S, McGrail K, et al. Exendin-4 therapy in NOD mice with new-onset diabetes increases regulatory T cell frequency. Ann N Y Acad Sci. 2008 Dec; 1150:152–156.
68. Hogan AE, Tobin AM, Ahern T, Corrigan MA, Gaoatswe G, Jackson R, et al. Glucagon-like peptide-1 (GLP-1) and the regulation of human invariant natural killer T cells: lessons from obesity, diabetes and psoriasis. Diabetologia. 2011 Jul 9; 54(11):2745–2754.
69. Makdissi A, Ghanim H, Vora M, Green K, Abuaysheh S, Chaudhuri A, et al. Sitagliptin exerts an antinflammatory action. J Clin Endocrinol Metab. 2012 Sep; 97(9):3333–3341.
70. Hogan AE, Gaoatswe G, Lynch L, Corrigan M a, Woods C, O'Connell J, et al. Glucagon-like peptide 1 analogue therapy directly modulates innate immune-mediated inflammation in individuals with type 2 diabetes mellitus. Diabetologia. 2013 Dec; 57:781–784.
71. Hadjiyanni I, Baggio LL, Poussier P, Drucker DJ. Exendin-4 modulates diabetes onset in nonobese diabetic mice. Endocrinology. 2008 Mar; 149(3):1338–1349.
72. Zhang J, Tokui Y, Yamagata K, Kozawa J, Sayama K, Iwahashi H, et al. Continuous stimulation of human glucagon-like peptide-1 (7-36) amide in a mouse model (NOD) delays onset of autoimmune type 1 diabetes. Diabetologia. 2007 Sep; 50(9):1900–1909.
73. ADA, Diagnosis and classification of diabetes mellitus. Diabetes Care. 2011 Jan; 34(SUPPL.1):62–69.
74. Roederer M. Compensation in Flow Cytometry. Curr Protoc Cytom. 2002 Dec;1.14.1–1.14.20.
75. Tauschmann M, Prietl B, Treiber G, Gorkiewicz G, Kump P, Högenauer C, et al. Distribution of CD4pos-, CD8pos- and Regulatory T Cells in the Upper and Lower Gastrointestinal Tract in Healthy Young Subjects. PlosOne. 2013;8(11):e80362.
76. Prietl B, Treiber G, Mader JK, Hoeller E, Wolf M, Pilz S, et al. High-dose cholecalciferol supplementation significantly increases peripheral CD4(+) Tregs in healthy adults without negatively affecting the frequency of other immune cells. Eur J Nutr. 2014 Apr;53(3):751-759.
77. Treiber G, Prietl B, Fröhlich-Reiterer E, Lechner E, Ribitsch A, Fritsch M, et al. Cholecalciferol supplementation improves suppressive capacity of regulatory T-cells in young patients with new-onset type 1 diabetes mellitus — A randomized clinical trial. Clin Immunol.; 2015;161(2):217–224.
78. Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes. 2005 May; 54(5):1407–1414.
79. Glisic-milosavljevic S, Waukau J, Jailwala P, Jana S, Khoo H-J, Albertz H, et al. At-Risk and Recent-Onset Type 1 Diabetic Subjects Have Increased Apoptosis in the CD4 + CD25 + high T-Cell Fraction. Apoptosis. 2007 Jan; 2(1):e146.

80. Hinman RM, Cambier JC. Role of B Lymphocytes in the Pathogenesis of Type 1 Diabetes. Curr Diab Rep. 2014;14(11):543.
81. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, et al. Administration of CD4CD25CD127 Regulatory T Cells Preserves b-Cell Function in Type 1 Diabetes in Children. Diabetes Care. 2012;35:1817–1820.
82. Leslie M. Regulatory T Cells Get Their Chance to Shine. Science. 2011;332:1020–1021.
83. Battaglia M, Stabilini A, Roncarolo M-G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood. 2005 Jun; 105(12):4743–4748.
84. Daniel C, Ploegh H, von Boehmer H. Antigen-specific induction of regulatory T cells in vivo and in vitro. Methods Mol Biol. 2011 Jan; 707:173–185.
85. Long SA, Rieck M, Sanda S, Bollyky JB, Samuels PL, Goland R, et al. Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs β-cell function. Diabetes. 2012 Sep; 61(9):2340–2348.
86. Gregori S, Giarratana N, Smiroldo S, Uskokovic M, Adorini L. A 1,25-Dihydroxyvitamin D3 analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice. Diabetes. 2002 May; 51(5):1367–1374.
87. Gorman S, Judge M a, Burchell JT, Turner DJ, Hart PH. 1,25-dihydroxyvitamin D3 enhances the ability of transferred CD4+ CD25+ cells to modulate T helper type 2-driven asthmatic responses. Immunology. 2010 Jun; 130:181–192.
88. Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, et al. Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. J Immunol. 2007 Nov; 179(9):6273–6283.
89. Adorini L, Penna G. Control of autoimmune diseases by the vitamin D endocrine system. Nat Clin Pract Rheumatol. 2008 Aug; 4(8):404–412.
90. Hyppönen E. Vitamin D and increasing incidence of type 1 diabetes-evidence for an association? Diabetes Obes Metab. 2010 Sep; 12(9):737–743.
91. Mohr SB, Garland CF, Gorham ED, Garland FC. The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide. Diabetologia. 2008 Aug; 51(8):1391–1398.
92. Munger KL, Zhang SM, O'Reilly E, Hernán MA, Olek MJ, Willett WC, et al. Vitamin D intake and incidence of multiple sclerosis. Neurology. 2004 Jan; 62(1):60–65.
93. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis Rheum. 2004 Jan; 50(1):72–77.
94. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266–281.
95. Toss G, Magnusson P. Is a daily supplementation with 40 microgram vitamin D3 sufficient? A randomised controlled trial. Eur J Nutr. 2012 Dec; 51:939–945.
96. Adorini L, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. Hum Immunol. American Society for Histocompatibility and Immunogenetics; 2009 May; 70:345–352.

97. Khoo A-L, Joosten I, Michels M, Woestenenk R, Preijers F, He X-H, et al. 1,25-Dihydroxyvitamin D3 Inhibits Proliferation but not the Suppressive Function of Regulatory T Cells in the Absence of Antigen-Presenting Cells. Immunology. 2011 Sep;134:459–468.
98. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. J Immunol. 2009 Nov; 183:5458–5467.
99. Hughson A, Bromberg I, Johnson B, Quataert S, Jospe N, Fowell DJ. Uncoupling of Proliferation and Cytokines From Suppression Within the CD4+CD25+Foxp3+ T–Cell Compartment in the 1st Year of Human Type 1 Diabetes. Diabetes. 2011; 60(8)2125–2133.
100. Gabbay M a L, Sato MN, Finazzo C, Duarte AJS, Dib S a. Effect of cholecalciferol as adjunctive therapy with insulin on protective immunologic profile and decline of residual β-cell function in new-onset type 1 diabetes mellitus. Arch Pediatr Adolesc Med. 2012 Jul; 166(7):601–607.
101. Bashir M, Prietl B, Tauschmann M, Mautner SI, Kump PK, Treiber G, et al. Effects of high doses of vitamin D3 on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. Eur J Nutr.; 2015 Jun; 55(4):1479-1489.
102. Thornton AM, Korty PE, Tran DQ, Wohlfert E a, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol. 2010 Apr;184(7):3433–3441.
103. Elkord E, Abd M, Samid A, Chaudhary B. Helios , and not FoxP3 , is the marker of activated tregs expressing GARP / LAP. Oncotarget. 2015;6(24):20026–20036.
104. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007 Oct; 87(4):1409–1439.
105. Kim W, Egan JM. The Role of Incretins in Glucose Homeostasis and Diabetes Treatment. 2008; 60(4):470–512.
106. Ban K, Noyan-Ashraf MH, Hoefer J, Bolz S-SS, Drucker DJ, Husain M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation. 2008 May 6; 117(18):2340–2350.
107. Dozier KC, Cureton EL, Kwan RO, Curran B, Sadjadi J, Victorino GP. Glucagon-like peptide-1 protects mesenteric endothelium from injury during inflammation. Peptides. 2009 Sep; 30(9):1735–1741.
108. Nyström T, Gutniak MK, Zhang Q, Zhang F, Holst JJ, Ahrén B, et al. Effects of glucagon-like peptide-1 on endothelial function in type 2 diabetes patients with stable coronary artery disease. Am J Physiol Endocrinol Metab 287. 2004 Dec; 287(6):e1209–15.
109. Arakawa M, Mita T, Azuma K, Ebato C, Goto H, Nomiyama T, et al. Inhibition of Monocyte Adhesion to Endothelial Cells and Attenuation of Atherosclerotic Lesion by a Glucagon-like. Diabetes. 2010 Apr; 59(4):1030–1037.
110. Kodera R, Shikata K, Kataoka HU, Takatsuka T, Miyamoto S, Sasaki M, et al. Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without

lowering blood glucose level in a rat model of type 1 diabetes. Diabetologia. 2011 Apr; 54(4):965–978.

111. Tashiro Y, Sato K, Watanabe T, Nohtomi K, Terasaki M, Nagashima M, et al. A glucagon-like peptide-1 analog liraglutide suppresses macrophage foam cell formation and atherosclerosis. Peptides. 2014 Apr; 54(April):19–26.
112. Noyan-Ashraf MH, Shikatani EA, Schuiki I, Mukovozov I, Wu J, Li R-KK, et al. A glucagon-like peptide-1 analog reverses the molecular pathology and cardiac dysfunction of a mouse model of obesity. Circulation. 2013 Jan; 127(1):74–85.
113. Krasner NM, Ido Y, Ruderman NB, Cacicedo JM. Glucagon-like peptide-1 (GLP-1) analog liraglutide inhibits endothelial cell inflammation through a calcium and AMPK dependent mechanism. PLoS One. 2014 Jan; 9(5):e97554.
114. Shirakawa J, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, et al. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. Diabetes. 2011 Apr; 60(4):1246–1257.
115. Berard M, Tough DF, Edward T. Qualitative differences between naive and memory T cells. Immunology. 2002;106:127–128.
116. Liberman A, Esser M, Marx N, Burgmaier M. Glucagon-Like Peptide-1 (9-36) Inhibits Chemokine-Induced Migration of Human CD4-Positive Lymphocytes. PLoS One. 2013; 8(3):e58445.
117. Eigler A, Siegmund B, Emmerich U, Baumann KH, Hartmann G, Endres S. Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production. J Leukoc Biol. 1998; 63(1):101–107.