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Immune profile
from high-risk to onset of Type 1 diabetes

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Thinking is more interesting than knowing,

but less interesting than looking.

Johann Wolfgang von Goethe

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ABSTRACT

Type 1 diabetes (T1D) is most often diagnosed early in life and is usually the result of an autoimmune attack on the insulin producing β-cells of the pancreas, leading to a lack of insulin secretion and life-long insulin treatment. The search for possible targets pin-pointing the β-cell destruction is a constant endeavour in the pursuit to prevent T1D onset. Hence, characterisation of the immunological profile and changes therein, during the pre-diabetic phase and disease course, is of outmost importance for the understanding of the immunological processes involved in T1D pathogenesis.

The aim of this thesis was to investigate the immunological profile, focusing on markers associated with T helper (Th) cells, pro-inflammation and regulatory T cells (Treg), in individuals with a high risk of developing T1D, and in children with newly diagnosed T1D for up to two years post diagnosis. In addition, we wanted to efficiently expand Tregs and detect any difference in T cell number and composition among T1D, high-risk and healthy individuals.

We found that high-risk individuals that later developed T1D had a lower mRNA expression of the regulatory associated markers forkhead box protein 3 (FOXP3), cytotoxic T lymphocyte associated antigen (CTLA)-4 and transforming growth factor (TGF)-β, following stimulation with the major autoantigen glutamic acid decarboxylase $(GAD)_{65}$, in combination with higher secretions of the chemotactic pro-inflammatory cytokine machrophage inflammatory protein (MIP)-1β, in comparison with high-risk individuals remaining undiagnosed.

In addition to a markedly altered immune profile during the pre-diabetic phase, T1D seems to present with an intense up-regulation of regulatory (FOXP3, TGF-β and CTLA-4) and pro-inflammatory (*e.g.* tumour necrosis factor-α) markers and a suppression of Th1 (*e.g.* interferon-γ) and Th2-associated immunity (*e.g.* interleukin-13). This up-regulation of regulatory markers, however, seems to occur too late in the immunological process to suppress the autoimmune attack directed against the pancreatic β-cells, and is probably reflecting the strong activation seen at onset of disease, rather than a cause of disease. Furthermore, we found low levels of circulating soluble CTLA-4 together with a positive correlation between soluble CTLA-4 protein secretion and mRNA expression in T1D, in parallel to a negative relation in healthy individuals. Moreover, low C-peptide was accompanied by low mitogen-induced soluble CTLA-4 protein, and vice versa, pointing to a link between clinical process, *i.e.* β-cell degradation and ability to secrete the regulatory molecule soluble CTLA-4 upon mitosis.

Our study also suggests that T1D children in our cohort were associated with a lower percentage of $CD4^+CD25^+CD127^{lo/-}$Tregs, however, the ones they had expanded well and even acquired a higher FOXP3 expression. We found an altered composition of $CD4^+$ subsets, biased towards a higher $CD4^+CD25^-$ ratio to Tregs.

In conclusion, the pre-diabetic phase seems to be accompanied by lower mRNA expression of regulatory associated markers in combination with higher secretions of the chemotactic pro-inflammatory cytokine MIP-1β, acknowledging the importance of studying this period in order to characterise the origin of T1D development. In addition, T1D seems to present with an intense up-regulation of regulatory and pro-inflammatory markers and a suppression of Th1 and Th2-associated immunity followed by low levels of circulating soluble CTLA-4 and, suggestively, lower percentage of $CD4^+CD25^+CD127^{lo/-}$Tregs. Whereas we found an altered composition of $CD4^+$ subsets, biased towards a higher $CD4^+CD25^-$ ratio to Tregs, the importance of said alteration remains to be shown.

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ORIGINAL PUBLICATIONS

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

I. **Rydén Anna,** Stechova Katerina, Durilova Marianna, Faresjö Maria

Switch from a dominant Th1-associated immune profile during the pre-diabetic phase in favour of a temporary increase of a Th3-associated and inflammatory immune profile at the onset of type 1 diabetes.

Diabetes Metabolism Research and Reviews 2009; 25: 335–343

II. **Rydén Anna** & Faresjö Maria

Altered immune profile from pre-diabetes to manifestation of type 1 diabetes.

Submitted

III. **Rydén Anna,** Bolmeson Caroline, Jonson Carl-Oscar, Cilio Corrado M., Faresjö Maria

Low expression and secretion of circulating soluble CTLA-4 in peripheral blood mononuclear cells and sera from Type 1 diabetic children.

Diabetes Metabolism Research and Reviews (accepted for publication)

DOI: 10.1002/dmrr.1286

IV. **Rydén Anna** & Maria Faresjö

Efficient expansion of cryopreserved CD4+CD25+CD127lo/- cells in Type 1 diabetes.

Results in Immunology 2011;1:36-44

ABBREVIATIONS

APC	antigen-presenting cell
APC	allophycocyanin
cDNA	complementary DNA
C-peptide	connecting peptide
CT	cycle threshold
CTLA-4	cytotoxic T lymphocyte associated antigen
DNA	deoxyribonucleic acid
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead box protein 3
FPG	fasting plasma glucose
GAD	glutamic acid decarboxylase
GADA	glutamic acid decarboxylase autoantibodies
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IA-2A	tyrosine phosphatase autoantibodies
ICA	islet cell autoantibodies
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon-inducible protein 10
MCP-1	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	machrophage inflammatory protein
mRNA	messenger- ribonucleic acid
MFI	mean fluorescence intensity
PB	pacific blue
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PerCP	peridinin chlorophyll protein complex
RANTES	regulated upon activation, normal T cell expressed, and secreted
RNA	ribonucleic acid
RT	reverse transcription
T1D	type 1 diabetes
T2D	type 2 diabetes
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
Treg	regulatory T cell
ZnT8	zinc transporter 8
ZnT8A	zinc transporter 8 autoantibodies

REVIEW OF THE LITERATURE

Type 1 diabetes

History of diabetes

As early as 1500 B.C, in the papyrus today known as Papyrus Ebers, the first clinical descriptions of diabetes were believed to be written. Later, in the 1st century AD, Aretaeus of Cappadocia described the clinical symptoms; "a melting down of the flesh and limbs into urine", of a disease he named diabetes. Diabetes was Greek for "runs through" and denoted the large urinary levels observed in patients. Even though ancient Chinese scripts describe the "honey urine" that took many lives, the word mellitus (Latin for honey) was not added to the name until the 17th century by the English doctor Thomas Willis. Dr. Willis had, as described long before by others, observed that the urine of patients was sweet (glycosuria). Due to these features of the disease, it was for more than two thousand years thought to be a disorder of the kidneys and bladder.

The causative factor of diabetes and the sweet urine, however, were not understood until 1889, when Joseph von Mering and Oskar Minkowski discovered that the removal of the pancreas in a dog led to development of diabetes mellitus and death. Joseph von Mering wanted to know what function this small organ might have in digestion but did not believe the removal of the organ would be a possibility in a live laboratory animal. Minkowski, on the other hand, thought otherwise and decided to remove the pancreas from a healthy dog. The dog, housebroken, soon started urinating on the laboratory floor even though taken out regularly. When tested, the urine contained high levels of sugar. The duo suggested that they had created diabetes with their experiment and inferred that the pancreas secretes a substance, later named insulin, which is involved with the body's use of sugar.

At this discovery, a myriad of experiments were initiated to try and isolate insulin. This however was a hard task, as the digestive enzymes produced by acini cells destroyed the insulin. A lot of researchers certainly tried, and a few succeeded, to isolate insulin, among them Oscar Minkowski. Successful isolation did, however, not become acknowledged, until 1921, when Frederick Banting and Charles Best blocked the pancreatic duct of dogs and thereby killed the acini cells prior to isolation of

insulin-containing extracts from the pancreas. This extract was given to diabetic dogs and immediately lowered their blood glucose levels and kept them alive significantly longer than their untreated counterparts. In 1922, a 14-year-old boy, severely affected by diabetes, was given regular insulin injections and amazingly regained good health. Even though insulin did not cure diabetes, it kept patients alive. A lack of insulin was now postulated as the cause of the disease.

During the 1930s, Harry Himsworth, a British clinician, discovered in both animals and humans, that not only the amount of insulin, but also the sensitivity to insulin, affected the body's use of sugar. This led him to believe that diabetes might not only be caused by a lack of insulin, but also by a lack of sensitivity to the same. By giving both sugar and insulin to diabetic patients, he found in some of the patients, a steep elevation of glucose in the blood, *i.e.* that some of the patients were insensitive to the actions of insulin. Diabetes showed not to be just one disease, but at least two types: type 1 (T1D) and type 2 (T2D). Type 1 often develops in the early years of the patients with an abrupt clinical onset, due to a lack of insulin production, while type 2 usually has a slower, more gradual onset that usually occurs in the middle ages and up. Patients affected by T2D are rather insensitive to insulin.

Definition and diagnosis of diabetes

Diabetes mellitus is a collective term describing not only one disease, but rather the outcome of several metabolic disorders with the characteristic features of hyperglycaemia and disturbances in the metabolism of fat, carbohydrates and protein. The development of diabetes is preceded by several pathogenic routes leading either to destruction of the insulin secreting β-cells, hence lack of endogenous insulin production, or to decrease of insulin responsiveness *i.e.* insulin insensitivity. Abnormal insulin secretion and insensitivity to its actions often coexists in patients [1, 2].

Classical symptoms leading to the suspicion of diabetes derive from hyperglycaemia, causing excessive thirst, polyuria, weight loss and sometimes recurrent infections and excessive hunger. In more severe cases, hyperglycaemia might be accompanied by ketoacidosis or a hyperosmolar state that is non-ketotic. These two states may lead

to coma or, in the worst case scenario, death if untreated. Retinopathy with risk of impaired vision or blindness, nephropathy and peripheral or autonomic neuropathy that might lead to foot ulcers, gastrointestinal and cardiovascular symptoms, are a few of the complications following long-term diabetes. Good glycaemic control is of utmost importance for preventing microvascular complications of long-term diabetes [3].

The American Diabetes Association recognizes three ways to set the diagnosis of diabetes, and as such one of the following criteria should be met: (i) fasting plasma glucose (FPG) above 7.0 mmol/l; (ii) classic symptoms of hyperglycaemia in combination with plasma glucose above 11.1 mmol/l independent of time of day; (iii) plasma glucose above 11.1 mmol/l, 2 hours after an oral glucose tolerance test (OGTT). The OGTT should be performed in accordance to the guidelines of the World Health Organization (WHO). When failing to show undisputable hyperglycaemia in FPG, at least 8 hours without caloric intake, or following OGTT, the test need to be confirmed on a different day [1, 2].

Children developing diabetes usually present an abrupt onset with severe clinical symptoms including strongly elevated blood glucose levels, increased thirst, glycosuria and ketonuria. Hence in children, diagnosis can often be confirmed and treatment started without the delay of blood glucose measurements or OGTT.

Even though hyperglycaemia is a classic symptom of diabetes, slight hyperglycaemia might be detected in connection with trauma, circulatory stress or acute infections. Hyperglycaemia under such conditions cannot be used as diagnostic ground, as it may well be transient. To set the diagnosis of diabetes, in cases like this or in individuals showing no symptoms, there is a need of at least one additional elevated plasma/blood glucose test.

Classification

Even though the majority of diagnosed cases of diabetes belong to the two major types, 1 and 2, there are in fact four classes based on aetiology.

Type 1 diabetes

This form of diabetes, the most severe one, was previously referred to as juvenile-onset diabetes, or insulin dependent diabetes, names that tell us a bit of the features of the disease. Type 1 diabetes (T1D) is most often diagnosed early in life and is usually the result of an autoimmune attack (Type 1 A diabetes) on the insulin producing β-cells of the pancreas, leading to a total, or an almost total, lack of endogenous insulin secretion. This means that the individuals affected will be in need of life-long insulin treatment for survival. In the majority of cases autoantibodies towards one or more pancreatic islet proteins, such as insulin (IAA), glutamic acid decarboxylase (GAD_{65}) or tyrosine phosphatases IA-2 and IA-2β, are present, indicating the autoimmune process. The β-cell destruction is usually rapid in young subjects and might be more prolonged in adults developing the disease, but the rate may vary greatly. The better retained endogenous insulin secretion the patient may have, the better chance they have to prevent ketoacidosis [2]. However, there are also idiopathic T1D cases, where autoimmunity does not precede the onset.

Type 2 diabetes

This is the most common form of diabetes and accounts for about 90-95% of all diabetes cases. The cause of impaired glucose uptake in these patients is foremost insulin resistance and often a relative insulin deficiency. As opposed to patients with T1D, patients with T2D rarely need insulin treatment for survival. Due to the milder form of hyperglycaemia and a slower progression, these patients may go undiagnosed for a very long time and even though symptoms are milder, there is an elevated risk of developing complications of various severities. T2D is strongly associated with obesity and a sedentary life-style, and as such this diagnosis typically occurs in adults even though the prevalence is increasing in children and adolescents. The increase in T2D diagnosis in a younger population is likely to be attributable to a sedentary lifestyle and increasing obesity which is more common now in that age group [4]. Obesity in itself causes some degree of insulin resistance, and as such weight loss alone can have a positive effect on glucose control.

The former strict discrimination between T1D and T2D is, however, becoming blurred as there are a growing number of cases with children and adolescents that seem to carry both types of diabetes. This expression of diabetes, now referred to as

double diabetes, usually presents as obesity and insulin resistance *i.e.* classical T2D features together with signs of pancreatic autoimmunity *i.e.* features of T1D [5, 6].

Gestational diabetes

Hyperglycaemia of various stages first discovered during pregnancy, will lead to the diagnosis of gestational diabetes. Gestational diabetes is usually discovered during the beginning of pregnancy to first half of the second trimester. Throughout this period the fasting and postprandial glucose concentrations are normally lower when compared to levels seen in non-pregnant counterparts. Postpartum, gestational diabetes often reverts [1, 2], but with an increased risk of developing T2D later in life.

Other specific types of diabetes

This group entails a variety of diabetes forms with different causative factors. Genetic defects of the β-cells might cause hyperglycaemia due to impairment in insulin secretion, while genetic mutations of the insulin receptor instead might lead to hyperinsulinemia and hyperglycaemia of various severities, due to insulin resistance. A number of hormones serve as antagonists of insulin, *e.g.* growth hormones, cortisol, glucagon and epinephrine, and any type of excess of such hormones may potentially cause diabetes. Beside genetic defects or endocrine abnormalities causing hyperglycaemia, injuries to the pancreas, no matter the injuring factor, also own the potential to cause diabetes [2]. Maturity onset diabetes of the young (MODY) is the collective name of a heterogeneous group of disorders characterised by β cell dysfunction. At least six different mutations have been associated with MODY. It has been estimated to be the cause of diabetes in 1-2% of children with diabetes. As optimal strategies for treatment are different for this group, it is important to distinguish MODY from T1D and T2D, however this is a hard task due to the clinical similarity [7].

Epidemiology of T1D

In the mid-1950s in North America and Northern Europe, the incidence of T1D started to increase in children and adolescents; currently an increase of 3-5% per year [8-11]. Before this time, childhood diabetes was uncommon, even though it was

probably underestimated in studies. Hence, when studying reports of prevalence and incidence before the 1950s one should keep in mind that factors such as cases unrecognized by the physician, early death due to lack of treatment and poor registration might have led to a great underestimation of the incidence.

Genetics is known to be strongly linked to the susceptibility of T1D; however the incidence increase rate seen today, is too rapid to be explained solely by genetics. Also, as T2D diagnosis are being delivered to a younger population at an alarming rate, it can be suspected that environmental factors, a more sedentary lifestyle, high caloric intake and poor nutrition in combination with genetic risk factors might influence the increase of both types. A multitude of hypotheses has been postulated to try and explain the global T1D increase. Popular hypotheses include the possible role of infections, increased body size, increased insulin resistance, the hygiene hypothesis and vitamin D exposure [12]. It has also been suggested that β-cell stress, such as seen during psychological stress, infancy and puberty, when β-cells have to produce large amounts of insulin, might be a risk factor for T1D development [13, 14].

Improved hygiene and living conditions resulting in a lower frequency of infections during childhood are suggested in the hygiene hypothesis, to be contribute to the increase in both autoimmunity and allergies [15]. There has been a change in antigenic exposure during the early years of childhood, altering the conditions for maturation of the immune system. Furthermore, the strong immune system built to defend against a lot of enemies (*e.g.* viruses, bacteria, worms etc) is not occupied, which may increase the risk of abnormal reactions. Finally certain virus may become so rare that immunity in the populations is too low. Studies showing negative association of T1D with factors such as having older siblings and lower economic status are used as arguments for this hypothesis [15].

Sweden has the second highest incidence of T1D in the world, reported as 45.3 and 41.9/100 000 for 2009 and 2010, respectively, in the 0-14.9 age group [16]. Only in our neighbouring country Finland, the yearly incidence is higher; 64.2/100 000 during 2005, in the same age group [17]. A high prevalence of risk-genes and a high life standard are common factors for the two Fennoscandian neighbours; supporting the

hypotheses presented in the previous sections as to why these countries may have these extreme rates.

Genetics

Today a complex interplay between non-genetic factors and a multiple genetic background is recognized as the risk of developing T1D. Currently four genetic loci are generally accepted as involved in the aetiology of the disease, even though about 40 loci have been connected to T1D risk in some way [18]. These are; allelic combinations of DQ-A1, DQ-B1 and DRB1 in the human leukocyte antigen (HLA, the equivalent to MHC) complex, variations in the insulin gene (INS), the cytotoxic T lymphocyte associated antigen-4 gene (CTLA-4) and the tyrosine phosphatase, non-receptor type 22 gene (PTPN22).

HLA

About half of the genetic/inherited risk of T1D is carried by the HLA complex region on chromosome 6p21, which indisputably make HLA the strongest T1D associated loci [19-21]. Three regions make up the HLA complex, class I, II and III (Fig. 1). The primary function of the HLA molecules is to protect against pathogens. While class I molecules present endogenous antigens, class II molecules present exogenous antigens to the T cell receptor on T cells, thereby initiating an immune response. In the 4 Mb long region wherein the HLA loci are encoded, about 200 genes have been identified. Of these genes, approximately one half are expressed and even though this region is the most crucial in adaptive and innate immunity, only a small proportion of these genes are involved in the immune response [19, 22]. The genetic set involved in immune responses encodes the classical HLA class I (A, B and C) and class II (DR, DQ and DP) antigens. These are all cell-surface proteins involved in the binding, and the presentation of antigens to T cells. Polymorphisms leading to conformational changes in the peptide binding groove of the cell-surface proteins, thereby altering the repertoire of peptides that can be bound and presented, are the most common polymorphisms in the HLA genes [19].

Polymorphisms of the HLA class II genes encoding DQ and DR are major determinants of genetic susceptibility to T1D, and to some extent polymorphisms of

the genes encoding DP (Fig. 1). A strong association is also seen for polymorphisms of the *HLA-B* class I gene. When comparing T1D children with a non-diabetic population for the frequency of HLA-DQB1 genotypes, its importance becomes apparent. Due to a strong link between HLA-DQA1 and DQB1, it is often sufficient to define the DQB1 allele and the DQA1 allele can be deduced on the basis of the common linkage disequilibrium [23].

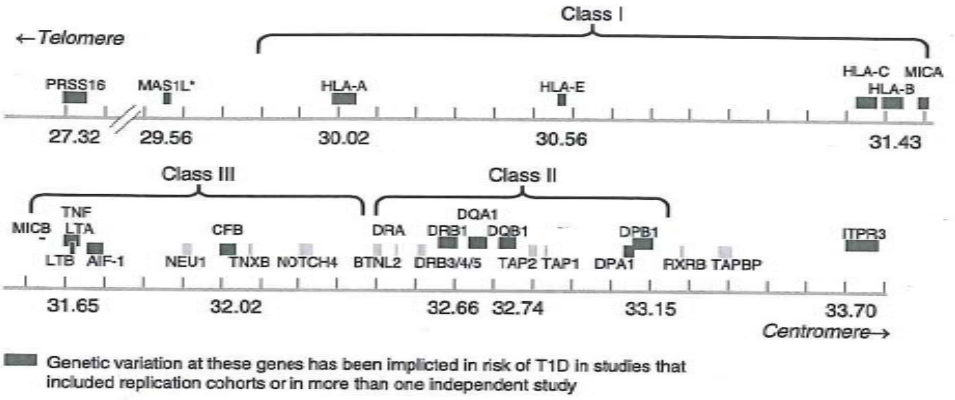

Figure 1 Schematic map of the HLA region.

Displaying genes within the HLA-region that have been associated with type 1 diabetes (T1D) Noble 2011 [19].

The DQB1*02 and *0302 alleles are associated with T1D risk, while the DQB1*0301, *0602 and *0603 are related to protection in a grading scale where different genetic combinations confer risk or protection to a certain degree [19, 23]. The heterozygous genotype commonly abbreviated as "DR3/DR4 is composed of the DRB1*0301-DQA1*0501-DQB1*02 on one chromosome and DRB1*0401/02/05/08-DQA1*0301-DQB1*0302/04 (or DQB1*02) on the other, and confers the highest risk of T1D. In the early nineties a study compared the genetics of Finnish and Greek children with T1D, representing the North and the South of Europe. While the DQB1*02 and *0302 alleles confer risk and DQB1*0301, *0602 and *0603 protection, in both populations, most Finnish children presenting with T1D are positive for the DQB1*0302, whilst Greek children with T1D to a higher extent are positive for DQB1*02 [24]. The DQB1*0602 is associated with dominant protection against T1D, even in combination with the DQB1*0302.

CTLA-4

At least two signals are needed for T cell activation; T cell receptor (TCR) signalling and a co-stimulatory ligand *e.g.* CD28. The CTLA-4 molecule is involved in negative regulation of T cell activation, competing with CD28 for the binding site on the B7 complex on antigen presenting cells (APC) (further information on page 29). T1D has been associated with the G allele in position 49 of the first exon, the so called +49A/G polymorphism. This allele is also associated with diminished control of T cell proliferation, which may be part of the explanation for association with T1D susceptibility [25]. Furthermore, homozygosity for the G allele is suggested to more than double the T1D risk [26]. The +49A/G polymorphisms have been connected to a potential reduction of soluble CTLA-4 in T1D patients carrying the G allele, in a gene dosage effect. Our group has previously shown that the CTLA-4 +49GG genotype was associated with lower percentages of intracellular CTLA-4 positive $CD4^{+}$ cells and tended to have lower percentages of intracellular CTLA-4 positive $CD25^{hi}$ (for regulatory T cells, read more on page 27) cells compared to AA genotype individuals [27].

INS

Polymorphisms in the insulin gene have been established as a susceptibility candidate. The polymorphism of interest leads to an alteration of the promoter region of the gene and thereby changes the expression of insulin in the thymus which ultimately plays its role in the deletion of insulin-specific autoreactive T cells [28, 29]. Hence, the T1D association with the INS gene might be due to mutations causing a decreased inulin mRNA expression and thereby a decreased removal of insulin-specific autoreactive T cells.

PTPN22

PTPN22 encodes the lymphoid-specific phosphatase (LYP) that is a very strong inhibitor of T cell activation performing its actions by dephosphorylating and inactivating T cell receptor-associated kinases and their substrates [30]. PTPN22 may also protect from apoptosis and cell death. A missense mutation in the PTPN22 gene, resulting in decreased effect of LYP on T cell activation, has been found to be strongly correlated with T1D incidence [31]. Further, an increased escape of

autoreactive T cells from thymus seems to be one of the features of mutation in the PTPN22 [32]. Both INS and PTPN22 might be associated with a pathway with insulin as a primary autoantigen.

Aetiology and Pathogenesis of T1D

T1D is caused due to pancreatic β-cell destruction in a process with autoimmune features, in genetically predisposed individuals, as mentioned previously. Hence, T1D is classified as an organ-specific autoimmune disease even though the pathological mechanisms leading to disease development are not fully understood. The autoimmune process leading to β-cell destruction, may be initiated several years before T1D diagnosis causing a proceeding decline in β-cell mass.

The symptoms of T1D often have an acute onset, which reflects the almost total lack of insulin secretion due to the great loss of β-cells. Most T1D patients develop acute ketoacidosis, coma and other serious metabolic complications without treatment, and are therefore dependent on insulin treatment for survival. At the onset of disease, the remaining islets usually contain degranulated β-cells, abnormal β-cells with enlarged nuclei and insulitis [33, 34].

The lifetime risk of developing T1D, among Caucasian populations, is approximately 0.4%, compared to 5-6% for the first-degree relatives of T1D patients (ref). In the case of a monozygotic twin affected by T1D, the disease develops in about 33% of the twins, while in dizygotic twins this number is only about 6%. This is consistent with a significant genetic contribution to T1D, as mentioned in the previous section. At the same time the high discordance rate among monozygotic twins indicates that the genes of susceptibility have low penetrance [35]

The high discordance rate among monozygotic twins and the rapid worldwide increase of T1D highlights the importance of environmental triggers, which may account for as much as two-thirds of disease susceptibility. Large differences in incidences, not simply attributable to genetic background further strengthens the theory of a multi-factorial background. E.g. although T1D associated susceptibility

HLA-DQ genotypes are equally distributed in Russian Karelia and neighbouring Finland, there is a nearly six-fold gradient in the incidence of T1D between the both populations [36].

Infection might trigger islet autoimmunity, and several studies have identified virus within isolated human islets from individuals with T1D, even though it has been difficult to obtain evidence that this is typical. Viruses are believed to be able to cause islet autoimmunity, for example by molecular mimicry, when antigens from the infectious agent share epitopes with an islet antigen. For this mechanism, the sequence shared between a section of GAD_{65} and a peptide of the Coxsackie B virus P2-C protein, is a widely known candidate [37]. Isolation of Coxsackie virus from the pancreatic islets of a child who died at T1D onset were done in a study and then *in vitro* cultured in rodent islets. This study demonstrated that the virus could then cause T1D in a rodent model [38]. Other studies of isolated adult human pancreatic islets has revealed that several different strains of Coxackie virus can infect human β-cells [39].

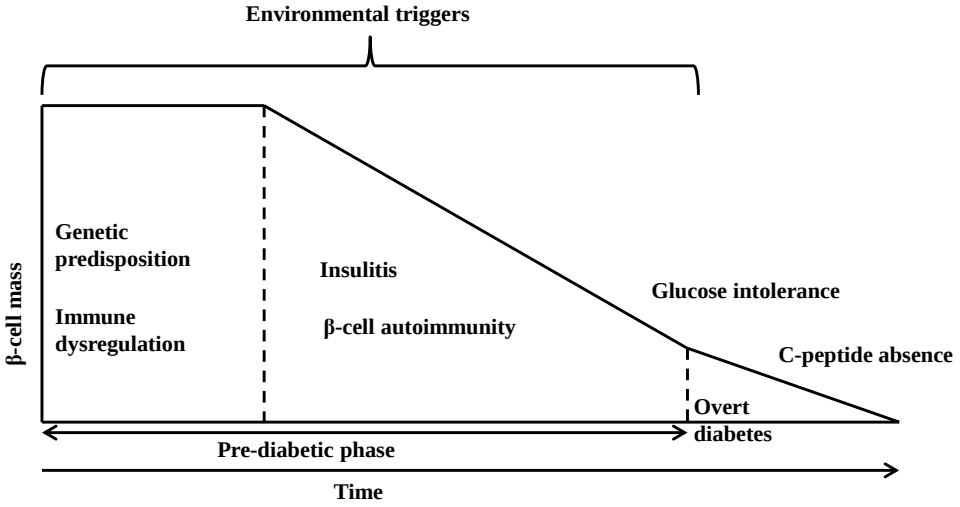

Figure 2 Hypothetical stages of the development of the multi-factorial disease T1D

A genetic risk predisposes for type 1 diabetes (T1D), however a triggering event is needed for development of the disease. The triggering event, leading to destruction of pancreatic β-cells, could be a virus infection. When 80-90% of all β-cells have been destroyed, clinical onset of type 1 diabetes takes place (modified from Atkinson 2001 [40].

Thus, both genetic and exogenous factors seems to contribute to T1D development and an assumption is that the autoimmune process preceding disease is triggered by several exogenous stimulus and occurs primarily in individuals carrying a genetic susceptibility to T1D (Figure 2).

Immunology

Introduction to the immune system

We are born into this world with an immune system as naïve and uneducated as ourselves. As the ultimate safeguard to the surroundings and ourselves, the immune system needs proper education in order to acquire proper ways to respond, to defend the barriers. This education takes place in the meeting with self and foreign agents throughout life. To maintain self-tolerance along with a sufficient protection against the many threats encountered in everyday life, the immune system needs to keep a plastic balance between up- and down-regulating mechanisms.

Innate immunity

The immune system is a complex and ingenious machinery, composed of several lines of defence, conducted by leukocytes. A first line of defence against a variety of common microorganisms is provided by macrophages and neutrophils of the innate immune system. As bacteria or other microorganisms infiltrate the body for the first time, by breaking the epithelial barriers, they will encounter a battery of cells and molecules that possess the ability to up-regulate an innate immune response. The invading microorganisms might be removed by engulfment, performed by macrophages that recognizes and bind elements of the surface of the invader. Alternatively, the macrophages might secrete chemotactic cytokines; chemokines, that attract cells bearing receptors for the specific chemokine, initiating an inflammatory process. This process can also be triggered by activation of the complement system, a system composed of plasma proteins that activates a cascade of proteolytic reactions on bacterial cell surfaces, but not on host cells, to ensure these surfaces are recognized by phagocytic receptors on macrophages. Heat, pain, redness and swelling (*calor, dolor, rubor and tumor),* are common denominators of inflammation and reflect the inflammatory mediators effects on local blood vessels. Heat, redness and swelling are caused by the increased permeability of the blood vessels and thereby increased blood flow and leakage that is induced during

inflammation. Complement and cytokines are also important for the induction of leukocyte adhesion to the endothelium of blood vessels and thereby for their migration to site of infection. This migration is the cause of the pain associated with inflammation. In the initial phase of inflammation, the main cell type seen in the inflamed tissue is neutrophils, shortly followed by monocytes that will differentiate into macrophages. Both neutrophils and macrophages are attracted to the site of inflammation by surface receptors for complement and common bacterial elements and perform their duties by engulfing and destroying the invaders. Moreover, dendritic cells of both myeloid and lymphoid lineages are part of the innate immune system and are important in the induction of adaptive immune responses [41].

Adaptive immunity

When a pathogen in the inflammatory site is engulfed by an immature dendritic cell, an adaptive immune response is prompted. The uptake of a pathogen will cause the activation of the naïve dendritic cell to become a specialised antigen-presenting cell (APC). While the initial cell type of innate immunity, the neutrophil, has a life span of just a few days, the specialised phagocytes of the adaptive immune system are long lived and migrate from their tissues through the lymph to regional lymph nodes to encounter and interact with naïve lymphocytes. Even though the dendritic cells can destroy pathogens, this is not their primary role, but rather to take up antigens and present them to and activate T lymphocytes. Cytokines influencing both innate and adaptive immune responses are secreted by the activated dendritic cells, making them highly important playmakers on the midfield, deciding what defence/offensive strategy to take in response to infectious agents. While the innate immune responses to common pathogenic structures are highly important, they do not induce a long term memory and a lot of pathogens own the ability to overcome these responses. Constituents of adaptive immunity however, have the ability to specifically distinguish pathogens and also to develop into memory cells that will be ready to rapidly respond to a repeated infection of the same antigen.

Blood cells

All blood cells arise from a pluripotent hematopoietic stem cell in the bone marrow which produces all types of differentiated blood cells but also, for example, osteoclasts in bone. The blood cells can be divided in to red (erythrocytes) and white

blood cells (leukocytes) and hold largely varying traits. While all erythrocytes follow the same developmental pattern and perform their duties by transporting O_2 or CO_2 in the blood stream, the leukocytes can further be divided into granulocytes, monocytes and lymphocytes, as defined by their appearance under the light microscope.

Granulocytes contain cytoplasmic granules such as secretory vesicles and lysosomes of different character, and can further be divided into three subgroups; neutrophils, basophils and eosinophils. They only leave the circulation to enter sites of inflammation, where neutrophils will phagocytose bacteria, while basophils and eosinophils enter sites of allergic inflammation. Basophils resemble mast cells in their function, with the secretion of histamine, and eosinophils are thought to contribute in the defence against parasites.

Monocytes will mature into macrophages upon exiting the blood stream and entering the surrounding tissue, and together with neutrophils compose the "professional" phagocytes of the body. They both contain vesicles (lysosomes), for example containing highly reactive superoxide (O_2-) and hypochlorite (HOCl), that will fuse with the phagosomes created around the engulfed invaders and thereby contribute to their degradation. The macrophages however, are larger and more long lived then the neutrophils. They also have a role in the cleaning of many tissues, where they will recognise and remove damaged, senescent or dead cells. Moreover, monocytes give rise to dendritic cells.

Lymphocytes are major players in the immune system, responsible for the strong specificity provided by the adaptive immune responses. The lymphocyte group comprises two major cell classes; T- and B cells, that both contribute to immune responses of various features. While T cells contribute to a more cell mediated immunity, B cells perform their major functions through antibody production (see below).

T-cells

T cells develop in the thymus from a lymphoid progenitor with common B cells, after its migration from the hemopoietic tissue (bone marrow in adults). The thymus, as well as the hemopoietic tissues, is thus referred to as the *central* lymphoid organs. While most lymphocytes die here, some will develop and be able to migrate to *peripheral* lymphoid organs, *e.g.* lymph nodes, spleen and epithelium-associated lymphoid tissues in the gastrointestinal tract, where they will encounter and interact with foreign antigens presented by APCs, before differentiating into effector cells [42].

The predominant part of a T cell carries T cell receptors (TCR), which are important in their maturation and activation. TCR are located in the membrane of the cells and are essential for the induction of T cell responses, which are dependent on contact with antigens presented on the HLA molecules. The TCR are composed by an α and a β chain, disulphide-linked polypeptide chains that together resemble one arm of a Y-shaped antibody, with two immunoglobulin (Ig)-like domains on each arm; making up for the great diversity of TCR and thereby the impressive range of antigens that can be recognised [42].

To achieve a well-balanced T cell repertoire, T cells undergo positive as well as negative selection. During positive selection, T cells that are able to receive so called "survival signals" through contact between the TCR and self-peptide presented on HLA-molecules on epithelial cells with low but significant reactivity, are the ones that survive. If the cells instead respond with a very high affinity to this binding, they are deleted by APCs in the process referred to as negative selection [43]. At this stage most lymphocytes die, due to lack of TCR signals. Further signals are received by the lymphocytes from cytokines such as IL-7. Continuous survival signals (*e.g.* in the form of IL-7) and contact with self-peptide bound HLA are conferred to the mature T cells after their migration from the thymus. Linage commitment of T cells seems to be due, not only to the potential of the cell, but to the successive loss of different options for development, thereby driving the maturation in a certain direction [44].

B cells

Unlike T cells that escape the bone marrow for their maturation to take place in the thymus, B cells remain there and mature. However, like the T cells, B cells also exit the primary lymphoid organ upon maturation to circulate the blood stream and thereby enter the peripheral lymphoid organs where they will encounter antigens that trigger their progressed maturation into effector B cells. This process usually requires T helper (Th) cells. The effector B cells secrete antibodies as means of their protective immunological function. The B cell equivalent of the TCR is the membrane bound B cell receptor, a surface immunoglobulin with two roles in the B cell activation. When an antigen binds to the B cell receptor, a signal will be transferred directly to the interior of the cell, in a similar manner as for TCR signalling. Further, after binding an antigen, the B cell receptor will embed the antigen to the interior of the cell to be degraded and later presented on the cell surface, bound to Major histocompatibility complex (MHC) class II molecules. This complex might then be recognised by antigen specific Th cells and, together with co-stimulatory signals provided through the connection between CD40 on the B cell and CD40 ligand (CD154) on the Th cell, prompts them to release cytokines that in turn gives the B cell a signal to proliferate and its offspring to differentiate into antibody secreting cells, and memory B cells [41, 42]. Hence, the first antibodies produced by a B cell is not released, but expressed on the cell surface as a receptor. Following antigen activation, the released antibodies will have the same unique antigen-binding sites as the initial surface B cell receptors.

Antibodies

Antibodies are an essential feature of the immune system, so important that vertebrates die of infection if unable to produce them [42]. Antibodies are found in the serum and other body fluids, and their function is to bind invading pathogens as a way of inactivating them. The binding of antibodies to pathogens is also protective, as lymphocytes and complement are attracted to the site. Although one B cell produces antibodies with a unique binding repertoire, mammals are able to produce antibodies of millions of different amino acid sequences and can thus respond to more than 100 million antigenic elements (theoretical assumptions), even such ones that do not exist in nature [45]. There are several classes of antibodies but the basic structure of the Y-shaped molecules remains the same. The arms confer the specificity and versatility of the antibody while the stem is decisive of the faith of the

immune response that will be elicited; *i.e.* complement opsonisation that leads to lysis, enhanced phagocytosis or allergy.

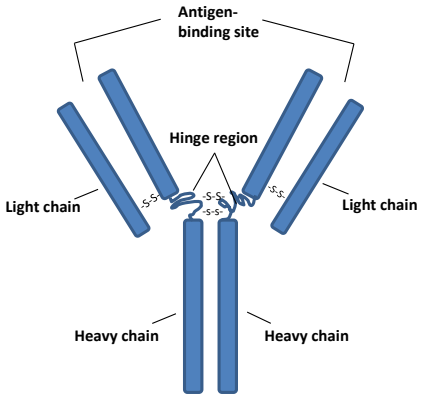

Figure 3 Schematic presentation of an antibody

The simplest form of an antibody is composed of two heavy chains and two light chains (adapted from Janeway [41]).

A typical antibody is Y-shaped and built up by two identical sets of polypeptide chains; two heavy and two light, that together make up for the two identical antigen binding sites at the end of the arms (Fig. 3). Antibodies can bind and crosslink several antigenic determinants and thereby create larger complexes that will easily be phagocytosed. Most antibodies have a flexible hinge region, between the stem and the fork of the Y-shape, which contributes to their efficient antigen binding and crosslinking. There are five groups, or isotypes, of antibodies in humans known as IgG, IgA, IgM, IgD and IgE, as defined by their different types of heavy chains. These all differ in characteristics and their function in the immune system. The most abundant antibody in the blood is the IgG, and accounts for about 80% of the antibodies in serum [41, 42, 45].

T cell subpopulations

As described previously, induction of T cell activation and maturation from the naïve T cell requires the TCR to encounter HLA class II bound antigen. This encounter will produce the first activation signal via CD3. To reach an optimal activation, however,

there is also a need for co-stimulation through interaction between the CD28 receptor on the naïve T cell and the CD80/CD86 complex (B7.1/B7.2) on the APC. This specific and relatively long-lasting interphase is commonly referred to as the immunological synapse. While the importance of CD28 costimulation during the initial activation of a naïve T cell has been widely accepted, it has now been shown that CD28 costimulation also has an important role for the reactivation of memory T cells to mount an ideal secondary T cell response [46]. Besides CD28; CTLA-4, inducible costimulator (ICOS), programmed cell death-1 (PD-1) and B- and T-lymphocyte attenuator (BTLA) have been described as costimulatory molecules. These molecules belong to the CD28 family and share structural homology, *i.e.* a variable, extracellular Ig-like domain together with a short cytoplasmic tail. Also costimulatory molecules belonging to the tumour necrosis factor (TNF)/TNFR (receptor) family have been described, such as the CD134 (OX-40), CD27 and glucocorticoid-induced TNFR-related protein (GITR) [46]. The effects of the costimulatory molecules might be enhancing the activation, as well as causing suppression.

T cells express the surface molecule CD3, and can be further divided into different subclasses based on their expression of CD4 and CD8. While naïve $CD4^+$ cells can differentiate into different Th cells (Th1, Th2, Th3, Th17, induced regulatory T-cells (iTreg) and follicular T helper cells (Fth)) upon encountering foreign antigens presented by APC [47], the $CD8^+$ cells make up the T cell family generally referred to as cytotoxic T cells (Tc), with the target-ligand HLA class I molecules, on infected cells.

Th1/Th2 paradigm

General

In the middle of the 1980s, two distinct subsets of $CD4^+$ T cells, producing distinctly different patterns of cytokines, were first described [48] and later confirmed [49, 50] in a mouse model. A few years later the concept of the $CD4^+$ subsets, named Th1 and Th2, were found to also be adaptive in the human T cell population [51, 52]. The Th1-dominated immune response can generally be said to be more aggressive and to contribute to cell-mediated immunity, with *e.g.* enhanced Tc activity and activation of macrophages, in the clearance of intracellular pathogens, such as viruses. The Th1-like cells perform their work, for example, by the production of interferon-γ (IFN-γ),

TNF-β and interleukin-2 (IL-2), and induces increased levels of inflammatory cytokines such as IL-1β and IL-6 [53]. In humans though, the synthesis of IL-2 is not as restricted to a single CD4+ T-cell subset as it is in a mouse model. Clones of the Th1 cells also induce delayed-type hypersensitivity (DTH) reactions, and IFN-γ is commonly expressed at sites of DTH reactions.

Humoral immunity connected to antibody production and enhanced eosinophil proliferation, is instead considered to be the activity of the Th2-like lymphocytes that produces IL-4, -5, -9 and -13, and protects from parasites. Th2 cytokines are commonly associated with strong antibody and allergic responses, if failing to control the production. Th2 responses are generally thought to be suppressive of Th1 responses and vice versa, showing a strong cross regulation between the phenotypes that inhibit the differentiation and effector functions of the reciprocal phenotype; *e.g.* IFN-γ inhibits proliferation of Th2 cells [50, 53-55], while IL-13 has been shown to suppress DTH responses [56]. Th1 cells have been found to be highly receptive to the natural regulatory T-cells (nTreg) suppressive functions, while Th2 cells seem to be poorly affected. This may depend on the Th2 cells ability to respond to other growth factors than IL-2, which has been proposed to be depleted from autoreactive T-cells by Tregs that may act as IL-2 sinks [57].

Even though the Th1/Th2 paradigm is still widely used and adaptable, synthesis of cytokines contributing to Th1 and Th2 patterns are associated with more than a single cell type, in human. Hence, one should keep in mind that the paradigm is an over-simplification, even if still useful. Different cell types seem to contribute either to the Th1 or Th2 profile, but there is a constant discovery of new Th cells (Th17 and iTreg) and cell types that will not easily fit into the model. It has also been shown that the $CD4^+$ T cell pool in humans might be immensely more plastic than we previously thought [47].

Chemokines

Chemokines is the collective name of a group of structurally related, small (~8-14 kDa) molecules, that will allow cells with an appropriate 7-transmembrane G protein-coupled receptor to migrate to the site of infection or tissue damage [58, 59].

Two major subfamilies based on the arrangement of the two N-terminal cysteine residues, CXC and CC, are used to classify chemokines. When there is an amino acid between the first two cysteine residues the nomenclature will be CXC and when neighbouring, CC. Furthermore, a numbering system based on the location of the gene encoding the chemokine is added to specify the cytokine [59, 60] (Table 1). For chemokine receptors, the same nomenclature followed by an R (receptor) is applied. The Th1- and Th2 subsets express different sets of chemokine receptors, which allow them to respond and migrate to different tissues [54], and the concept of Th1- or Th2 immunity can also be extrapolated to chemokine secretions and the expression of their receptors. Th cells express different sets of chemokine receptors. The interferon-inducible protein (IP)-10 receptor, CXCR3, is expressed by Th1 cells, while CCR3 binding CCL5 (regulated upon activation, normal T cell expressed, and secreted, RANTES) is foremost expressed by Th2 cells. RANTES, however also binds to the more promiscuous receptor CCR5 found to be expressed on both Th1 and Th2 cells [55, 61]. CCL3 (Machrophage Inflammatory Protein (MIP)-1α) and CCL4 (MIP-1β) also binds to the receptor CCR5 [61]. Despite the reported promiscuity of the CCR5 receptor, MIP-1α, MIP-1β and RANTES are often discussed in relation to Th1 immunity. Further, CCL2 (monocyte chemoattractant protein, MCP-1), has been suggested to be produced by Th1 cells [62]. Induction of Th1 and Tc activity has shown a strong association to the up-regulation of CXCR3, *e.g.* binding CXCL9, (monokine upregulated by IFN-γ, MIG) and CXCL10 (IFN-γ–inducible protein 10, IP-10) [63].

Table 1 Common name versus systemic nomenclature of chemokines, and examples of their receptors.

Human ligand	Systemic name	Chemokine receptor(s)
MIG	CXCL9	CXCR3
IP-10	CXCL10	CXCR3
MCP-1	CCL2	CCR2
MIP-1α	CCL3	CCR1, CCR5
MIP-1β	CCL4	CCR5
RANTES	CCL5	CCR1, CCR3, CCR5

Connection to disease

Autoimmune diseases have been connected to various cytokines and chemokines, both in respect to beneficial as well as aggravating effects. T1D have been connected

to a disequilibrium in the Th1/Th2 balance, with an overproduction towards Th1-associated cytokines that have been suggested to be pro-diabetic and enhance the autoimmune process [64]. Th1-like subtypes secreting dominantly IFN-γ and TNF-β have been shown to be important for the destruction of the insulin-producing β-cells [65, 66]. Pro-inflammatory cytokines, especially TNF-α and IL-6 and chemokines including MIG, MIP, MCP-1 and IP-10, have shown proof to home to the inflammatory site [67]. In contrast, Th2-like cytokines *e g* IL-4, IL-5 and IL-13 have been shown to be down-regulated during this organ specific autoimmune process [68-70].

Regulatory T-cells

Subgroups/identification

Regulatory T cells (Tregs) are thought to be responsible for maintenance of self-tolerance and immune homeostasis and can be divided into naturally occurring (nTreg) and induced phenotypes (iTreg). nTregs are derived in the thymus as a consequence of natural selection and give rise to a long-lived endogenous population of self-antigen specific T-cells in the periphery. Their principal function is to prevent autoimmunity, but will also suppress many immune responses [71]. They express CD4, high levels of CD25 ($CD4^+CD25^{hi}$), CTLA-4, Forkhead box protein P3 (FOXP3) and the glucocorticoid-induced tumour necrosis factor receptor (GITR). However, all of these markers are also up-regulated upon stimulation of effector CD4+ T cells [72]. Tregs can also be identified by the combination of CD25 and FOXP3 expression and lack of, or low, expression of CD127, the IL-7 receptor α chain, which also has been shown to correlate with suppressive function [73, 74]. Furthermore, activated Tregs have been distinguished from resting Tregs due to the absence of CD45RA in combination with high FOXP3 expression, as opposed to low FOXP3 expression in combination with CD45RA expression in resting Tregs [75].

Antigen-specific subset populations of $CD4^+$ iTregs, in contrast to $CD4^+CD25^+$ Tregs, develop in the periphery in response to activation of mature T cells and depend on cytokines for development. The induced Treg cells will, contrary to natural Treg cells, suppress only certain immune responses via cell-cell contact or by suppressive cytokines. The adaptive regulatory cells include the IL-10-producing T-regulatory cell type 1 (Tr1) [76], secreting IL-4 and IL-10, and transforming growth factor (TGF)-

β-secreting Th3 cells [77]. TGF-β is an important marker of Tregs of an adaptive nature, expressed after activation [57]. The main suppressive mechanism of Th3 cells is dependent on the production of TGF-β, which suppresses the proliferation of Th1 and Th2 cells. Interleukin-10, with the concurrent inhibition of IL-12, may also augment the expansion of Th3 cells by decreasing the development and maturation of Th1-like cells, which in contrary can inhibit Th3 expansion [77].

In mouse models completely deprived of Tregs, a variety of autoimmune diseases develop. Furthermore, a variety of diseases and autoimmune states have been associated with impaired Treg function [78-82], while reintroduction of $CD4^{+}CD25^{hi}$ Treg cells into non-obese diabetic (NOD) mice has shown to prevent the development of T1D and to inhibit the production of IFN-γ [83]. It has proven difficult to generate any unequivocal answers as to the role of Tregs in human autoimmune disease, and data presented have often been contradictive. However, the collective data suggest that there are defects in Treg function or numbers in various autoimmune disorders, even though this might differ between different states and individuals due to the great heterogeneity of the immune system. While systemic lupus erythematosus (SLE) seems to be more related to defects in Treg numbers, multiple sclerosis (MS) seem to be more often associated with decrease in Treg function. In addition, T1D has been suggested to be connected to Teffs resistant to Treg suppressive effects (as reviewed in [84]).

FOXP3/CTLA-4/sCTLA-4/TGF-β

FOXP3

FOXP3 is considered to be a key regulator of Treg development and function [85] and mutations in humans are known to cause X-linked syndrome (IPEX) associated with high incidences of autoimmune diseases [78, 79]. Ravaging disease associated with FOXP3 deficiency is due to Treg scarcity [86]. Even though the role and function of FOXP remains tentative, it has proven to be very important for Tregs.

TGF-β

TGF-β is a regulatory cytokine involved in the control of proliferation, differentiation, and other T cell functions. It has also been shown to induce apoptosis

in various cell types. Previously, TGF-β was considered to be important for nTreg commitment, but was recently suggested to instead antagonize the negative T-cell selection and in this way promote nTreg survival and thereby control autoreactive T-cells [87]. In addition, a deficiency of TGF-β has been associated with fatal inflammatory diseases in murine models [88].

Full length and soluble CTLA-4

FOXP3+ Tregs constitutively express CTLA-4 [89], while other CD4+ T-cell subsets predominantly express the protein in intracellular vesicles and upregulate it transiently upon activation [90]. CTLA-4 expression, both intracellular and extracellular, has been shown to be higher on Tregs than their CD4+ and CD4+CD25- counterparts [91]. CTLA-4 has been shown to be essential for the maintenance of immune homeostasis, and *in vivo* blockage of CTLA-4 on CD4+CD25+ Tregs has been shown to induce organ specific autoimmunity in mice [92]. In addition, strong CD28 costimulation suppresses induction of iTreg from $CD4^{+}CD25^{-}$ T cells [93], while costimulation of CD28 is critical in the thymic development of natural Tregs and their peripheral maintenance [94] and have been suggested to be required in low levels for iTreg generation [95]. However, while CD28 seems to be dispensable for the functional re-activation of Tregs, CTLA-4 might be required, as its blockage abrogates suppression of T cell responses while Tregs deficient of CD28 still can cause suppression. Tregs may, at least in part, exert their immunosuppressive function through CTLA-4 [96], by binding of the B7 complex (CD80/CD86) on antigen-presenting cells (APCs) with higher affinity than CD28 (Fig. 4) [97, 98]. This leads to increased tryptophan catabolism, thereby conferring an inhibitory immune signal down-regulating T effector (Teff) activity and causing poor T cell proliferation responses [96, 99].

The CTLA-4 gene seems to yield at least two transcripts, one encoding a full length transmembrane protein that binds the B7 complex on APCs [100] and a second that seems to encode the soluble form of CTLA-4, lacking the transmembrane domain encoded by exon three [101]. Recombinant soluble CTLA-4 immunoglobulin has been shown, both *in vitro* and *in vivo*, to inhibit T-cell proliferation; for example in co-cultures of T-cells and B7-expressing APCs [97, 102-104]. Currently, due to its immunosuppressive effect, soluble CTLA-4 immunoglobulin is used to treat autoimmune disorders such as rheumatoid arthritis [105]. At the same time, several

studies have shown increased levels of soluble CTLA-4 to be associated with autoimmunity [101, 106-110]. Hence, while the full length product of CTLA-4 indisputably contributes to down-regulation of T cell activation, the role of soluble CTLA-4 in the immune system is more disputed.

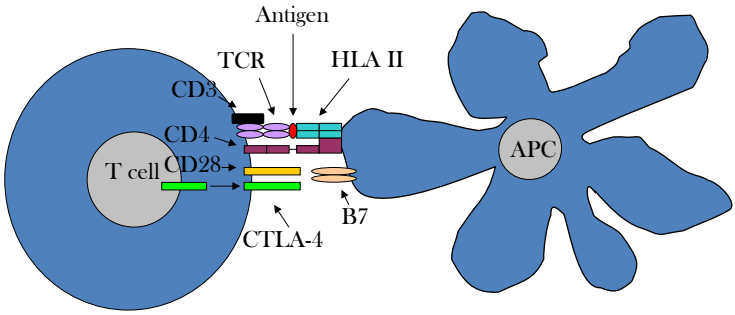

Figure 4 The immunological synapse.

The T cell encounters an antigen presenting cell (APC) with antigen peptide presented on its MHC (HLA) class II molecule. The T cell will recognise the HLA-peptide complex through the T cell receptor (TCR), which will then induce the first activation signal via CD3. The T cell will thereafter receive a second and important signal through the binding of CD28 to the B7 complex (composed of CD80/CD86). Following activation, up-regulation of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) takes place and outcompetes the CD28 binding to B7 and thereby breaks the activation signal, which consequently confers an inhibitory signal.

Autoantigens and autoantibodies

Autoantigens are the classification of antigens that trigger an immune response to self. In the pathogenesis of T1D, several autoantigens have been indicated due to the presence of autoantibodies. Detectable autoantibodies are the first signs of evolving β-cell immunity and can be present years before clinical onset of disease. Four autoantibodies associated with T1D, have previously been shown to predict clinical T1D development [111]; islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies towards the 65kD isoform of glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-related IA-2 molecule (IA-2A). Also antibodies towards the zinc transporter 8 (ZnT8) have recently been associated with the prediction of T1D [112]. The presence of multiple autoantibodies is indisputably related to the risk of progression to manifest T1D, and have been shown both in family studies and in the general population [113, 114]. While approximately 95% of

T1D patients express a minimum of one autoantibody and 80% at least two autoantibodies [115, 116], only 1% of the healthy population displays autoantibodies against GAD_{65} or IA-2 [116, 117].

Insulin

Autoantibodies against insulin (IAA) was first described in newly diagnosed T1D children in 1983, and as such insulin was the first β-cell antigen to be shown [118]. The first, or among the first, autoantibodies to appear during the pre-diabetic phase in children are usually IAA, and they are often detected earlier than the other autoantibodies [119]. These autoantibodies have also been shown to be strongly related to the T1D risk-associated HLA DR-4DQ8 haplotype [120]. Approximately 40-70% of T1D patients, depending on age group and region, are positive for IAA [121, 122], while only about 2.5% of healthy children display IAA [116]. However, it has been suggested that T1D patients positive for IAA display a clinically milder disease [122].

Insulin is synthesized from a precursor molecule (proinsulin) consisting of three segments, the B, C and A sequence. During the processing of proinsulin to insulin, the mid-segment, the C-peptide is removed. The remaining B- and A-chains, are coupled together by disulphide bonds. Equimolar amounts of C-peptide and insulin are stored in secretory granules of the β-cells. As C-peptide is cleaved of and also released into the circulation, it is used as a marker of endogenous insulin secretion and β-cell function.

Glutamic acid decarboxylase (GAD)

Glutamic acid decarboxylase (GAD) is an enzyme responsible for the biosynthesis of γ-aminobutyric acid (GABA). It is found as a 67kDa (GAD_{67}) and a 65kDa (GAD_{65}) isoform in humans, encoded by the *Gad1* and *Gad2* genes, respectively [123]. The *Gad1* and *2* are both expressed in the brain, while only the *Gad2* appears to be expressed in the pancreatic β-cells in humans [124]. Although GAD and GABA have an important role as an inhibitory neurotransmitter in neurons, their role in the pancreas is still largely unknown. The increase of GAD_{65}, and thereby GABA, have

been studied and suggested that the action of the transmitter might be to regulate, or impair, the first phase of the insulin secretion induced by glucose [125].

GAD_{65} is a putative autoantigen in T1D and autoantibodies towards it (GADA) have been shown to be strongly correlated with HLA-DR3-DQ2. GADA have also been shown to be more frequent in females [120]. About 2% of healthy children are positive for GADA [116] in contrast to reports of 50-80% of T1D patients [117, 122].

Tyrosine phosphatase-like protein

The tyrosine phosphatase like protein islet antigen-2 (IA-2) is a transmembrane protein, expressed in secretory granules of human pancreatic islet cells, and also in other peptide-secreting endocrine cells and neurons [126]. IA-2 has been identified as a major autoantigen in T1D [127, 128]. When sera from T1D patients and healthy controls were tested for reactivity towards IA-2, it was found that 66% of T1D patients but none of the healthy controls reacted with IA-2 [127]. Similarly, up to 88% of newly diagnosed T1D patients have tested positive for autoantibodies towards IA-2 (IA-2A), while only 0-2.5% of the general population reacted positively [117, 126]. IA-2A has been suggested to be a more precise marker in the identification of β-cell autoimmunity than the other autoantibodies. This could be due to a strong association with the HLA-DR4-DQB1*0302 and the lowest C-peptide concentrations seen at diagnosis [120].

Zinc transporter 8

The zinc transporter 8 (ZnT8) is a transmembrane spanning protein exclusively transcribed in the pancreas, primarily in the Islets of Langerhans with the highest expression in β-cells. The ZnT8 aids accumulation of zinc from the cytoplasm to intracellular vesicles and might be important in providing zinc to maturation and/or storage of insulin in β-cells [129]. The ZnT8 was recently described as a major autoantigen in human T1D [112]. The ZnT8 was found to be targeted by autoantibodies (ZnT8A) in 60-80% of new-onset T1D patients, in comparison to less than 2% of controls and less than 3% of patients with T2D. Moreover, ZnT8 was found in 26% of T1D individuals that had previously been described as autoantibody negative. When individuals were followed from birth until clinical T1D onset, ZnT8A

was detected as early as two years of age and was persistent during the pre-diabetic phase, however, it commonly appeared after GADA and IAA [112]. The ZnT8 has not only been suggested as a major autoantigen in T1D due to the presence of autoantibodies, but also due to finding specific autoreactive T cells targeting the protein in human T1D [130].

European Nicotinamide Diabetes Intervention Trial (ENDIT)

Nicotinamide is a water-soluble vitamin, also known as niacinamide or nicotinic acid amide and is the amide of nicotinic acid. Nicotinamide belongs to the vitamin B group (vitamin B_3) and has been classified as a food additive. *In vivo,* nicotinic acid, or niacin, is converted to nicotinamide. Nicotinic acid and nicotinamide share the same vitamin functions but nicotinamide does not have the side-effects of nicotinic acid, *e.g.* vasodilation (flushing), itching and burning sensations. Nicotinamide makes up the operative part of the coenzyme nicotinamide adenine dinucleotide (NAD), important in numerous oxidation-reduction reactions [131]. When deoxyribonucleic acid (DNA) of cells is damaged, enzymes working to repair DNA will be activated, a process consuming NAD. This process will drain the intracellular energy storages and in cases of strong activation, lead to cell death. It has been known for a long time that pre-treatment with high doses of nicotinamide prevents diabetes development in rats treated with streptozotocin and also prevents, or delays, onset in NOD mice. Nicotinamide, in addition to insulin, at onset of T1D has shown to offer some prolongation of retained residual β-cell function in humans [132] and also prevents or delays T1D development in ICA positive children in New Zealand [133]. It has been suggested that high doses of nicotinamide could modify cell death pathways in β-cells, *e.g.* by affecting this process and restoring the NAD levels. This would, hence, lead to improved β-cell survival [134].

The ENDIT study was a randomised, double-blind, placebo-controlled trial, aimed at preventing β-cell destruction and thereby preventing or delaying T1D, through delivery of high dose nicotinamide. First-degree relatives of T1D patients that had developed the disease before their 20th year were screened for the presence of ICA levels. Juvenile Diabetes Federation (JDF) units as high as 20 or more are postulated to correlate with a risk of up to 40% for developing T1D within five years and was criteria for inclusion. More than 30 000 first-degree relatives were screened in order

to recruit 552 study subjects from 18 European countries and also from Canada and the USA. They were randomly assigned oral nicotinamide (Ferrosan AC, Copenhagen, Denmark) at a dose of 1.2g/m^2 daily (maximum 3g/day) or a placebo treatment for five years [135]. In Sweden, more than 2000 first-degree relatives of T1D patients were screened to identify high-risk individuals meeting the set criteria; twenty-three of those were included in the trial. In the Swedish arm of the trial, seven of the twenty-three high-risk individuals developed T1D already during the study. The ENDIT study did not show any differences in development of T1D between nicotinamide and placebo treated subjects, which was proposed to be due to ineffective dosage [135].

AIMS OF THE THESIS

The general aim of this thesis was to investigate the immunological profile in individuals with a high risk of developing T1D, and in children with newly diagnosed T1D for up to two years post diagnosis.

Specific aims:

I. To **confirm previous observations of a dominant Th1-like profile** during the period prior to onset of disease. Further, to follow the **immune response for up to two years**, focusing on the spontaneous as well as autoantigen induced immune profile in comparison with high-risk and healthy children.

II. To investigate the **composition and possible changes of the immunological profile**, both spontaneously and following stimulation with the putative autoantigens GAD_{65} and HSP_{60}, from high-risk to T1D onset and six months post diagnosis.

III. To study whether there were any **differences in soluble CTLA-4 and its relation to full length CTLA-4 and other Treg associated markers.** Thus, soluble CTLA-4 and full length CTLA-4, together with FOXP3 and TGF-β, were studied at three time points in T1D children as well as in individuals with high or low risk of developing the disease.

IV. To investigate the **cryostability of Treg associated markers and subsequently sort and expand Tregs** from cryopreserved PBMCs of T1D, high-risk and healthy individuals. The aim was also to efficiently expand Tregs and to detect any difference in T-cell number and composition among the studied subjects.

SUBJECTS AND METHODS

Study groups

In this thesis four different study populations were included; individuals with such a high risk as 40%, for developing T1D within five years, previously included in the ENDIT study (see section "European Nicotinamide Diabetes Intervention Trial (ENDIT)"), T1D children enrolled at the paediatric diabetes clinic at the Linköping University Hospital, healthy school children, and healthy adults. Blood samples were collected from these four study cohorts, when possible, during the morning hours to avoid time-of-day differences.

High-risk individuals

First-degree relatives (both children and adults) of T1D patients that had a postulated risk of up to 40% (≥ 20 ICA IJDF units) for developing T1D within five years participated, as described previously, in the ENDIT study. They were randomly assigned oral nicotinamide (Ferrosan AC, Copenhagen, Denmark) at a dose of 1.2g/m^2 daily (maximum 3g/day) or a placebo treatment for five years [135]. Blood samples from these high-risk individuals were transported to Linköping within 24 hours of blood sampling.

T1D children

Blood samples from children with T1D were taken during routine visits to the paediatric diabetes clinic at the Linköping University Hospital. Patients were asked to give one additional blood sample for research purposes and all children and/or their parents have given their informed consent to participate. Samples were collected at 4-10 days, approximately 6 months, one and two years post T1D diagnosis.

Healthy control subjects

Blood samples from healthy children were collected through school healthcare. All children and/or their parents have given their informed consent to participate. The children were asked to fill out a questionnaire regarding their family health, together

with their parents. The questions regarded presence of T1D, T2D, celiac disease, rheumatoid arthritis, goitre and allergies in first-degree relatives and themselves. Children affected, or with a first-degree relative affected, by any of the mentioned conditions, or with an on-going infection were excluded from participating.

From healthy adults (paper IV), blood samples were collected at the work place. These healthy adults did not have any on-going infections or family history of T1D. All individuals gave their informed consent.

Paper I and III

For paper I and III, the same study subjects were used; T1D children (at four days, one year and two years post diagnosis), high-risk individuals, and healthy children (table 2). Healthy control children included in paper I and III carried the HLA genes DR4-DQ8 (DR4-DQA1*03-DQB1*0302), DR3-DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) DR13-DQ6 (DRB1*1301-DQB1*0603) or DQ1302-DQ6 (DRB1*1302-DQB1*0604) in different combinations. In figure 5, the distribution of HLA genes between the healthy controls and high-risk individuals can be seen.

Table 2 Study subjects of paper I and III

T1D	Age	Gender	Healthy	Age	Gender	High-risk	Age	Gender	Treatment	Developed T1D
			No							time after sampling
T1D1	11	Female	HC1	7	Female	HR1	8	Female	Nicotinamide	Yes (6 months)
T1D2	11	Female	HC2	9	Female	HR2	12	Female	Placebo	Yes (12yrs)
T1D3	12	Female	HC3	10	Female	HR3	10	Male	Placebo	No
T1D4	14	Male	HC4	15	Female	HR4	12	Male	Nicotinamide	Yes (4 yrs)
T1D5	11	Female	HC5	7	Male	HR5	14	Male	Nicotinamide	Yes (12 months)
T1D6	12	Female	HC6	11	Male	HR6	15	Male	Nicotinamide	No
T1D7	16	Female	HC7	13	Male	HR7	15	Male	Placebo	No
T1D8	5	Male	HC8	14	Male	HR8	18	Male	Placebo	Yes (9 yrs)
T1D9	7	Male	HC9	12	Male	HR9	22	Male	Placebo	No
T1D10	12	Male	HC10	9	Female					

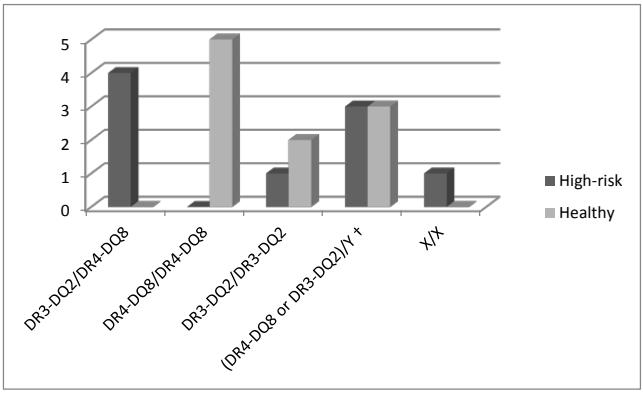

Figure 5 *Distribution of HLA genes in healthy controls and high-risk study subjects in paper I and III*

Paper II

In paper II, the study population comprised T1D children (at 4-10 days and 6 months post diagnosis), high-risk individuals, and healthy control children (table 3).

Table 3 ***Study subjects paper II***

No	Age	Gender	HLA	S1	S2	Age	Gender	HLA	Age	Gender	HLA	IA-2A	GADA	IAA	Treatment	DevT1D time after sampling
1	3	Male		No	9 mon	8	Female	301,0302	11	Male	0302	0	0	0	Placebo	No
2	4	Female	201,0302	No	8 mon	8	Female	2/5	12	Male	0201	2	2	0	Nicotinamide	Yes (4 yrs)
3	4	Female	501,0602	No	10 mon	8	Male	302,0602	16	Male	0201/0302	0	2	1	Nicotinamide	No
4	4	Female	0201/0501	No	6 mon	9	Male	301,0603	17	Female	0302	0	2	1	Placebo	Yes (9 yrs)
5	4	Male	402	No	5 mon	9	Male	301,0603	18	Male	0201/0602	0	2	1	Placebo	No
6	4	Male		No	10 mon	10	Female	301	21	Male	0201/0201	2	2	0	Placebo	Yes (6 yrs)
7	4	Male		**Yes**	9 mon	13	Female	301	26	Male	0201/0201	2	2	0	Placebo	No
8	5	Female	0302/030	**Yes**	10 mon	13	Male	301,0603	33	Female	0201/0302	2	2	0	Placebo	Yes (13 yrs)
9	6	Male	302	**Yes**	7 mon	13	Male	301,0602	41	Female	0302/0302	1	2	2	Nicotinamide	Yes (11 yrs)
10	7	Male	201,0302	**Yes**	6 mon	14	Female	301,0603	43	Female	0201/0302	2	2	0	Nicotinamide	No
11	8	Female		No	9 mon	14	Male	X	43	Female	0302/0302	2	2	2	Nicotinamide	Yes (1 y)
12	9	Female	20,0302	No	9 mon	15	Female	2,0602/5	47	Male	0201	0	2	0	Nicotinamide	Yes (11 yrs)
13	10	Male	604,0201	No	9 mon	15	Female	2/5	48	Male	0201/0302	0	0	0	Nicotinamide	No
14	11	Male	0501,060	**Yes**	8 mon	15	Female	0602	48	Female	0201	0	0	0	Nicotinamide	No
15	11	Female	302	No	9 mon	15	Male	301	53	Female	0302	0	2	0	Nicotinamide	No
16	11	Female	6,0302	No	6 mon	15	Male	0602								
17	12	Female	302,0501	No	9 mon											
18	12	Female	0302/0305	No	9 mon											
19	13	Male	02,0302	**Yes**	9 mon											
20	14	Male		**Yes**	10 mon											
21	15	Male	501,0302	No	9 mon											
22	16	Female	302	**Yes**	7 mon											
23	16	Female		**Yes**	6 mon											
24	16	Female	201,0302	**Yes**	5 mon											
25	17	Male	302,04	No	10 mon											

Paper IV

In paper IV, the study group were composed of T1D children (approximately 1-10 months post diagnosis), high-risk individuals, and healthy control individuals as seen in table 4.

Table 4 Study subjects of paper IV

T1D	Duration	Age	Gender	Healthy	Age	Gender	High-risk	Age	Gender	Treatment	Developed T1D	
				No								
T1D1	3 months	4	female	HC1	9	female	HR1	17	female	Placebo	yes	8.5yrs post sampling
T1D2	6 months	11	female	HC2	13	male	HR2	41	female	Nicotinamide	yes	10yrs post sampling
T1D3	9 months	10	male	HC3	8	male	HR3	12	male	Nicotinamide	yes	4yrs post sampling
T1D4	5 months	4	male	HC4	15	male	HR4	42	female	Placebo	yes	1.5yrs post sampling
T1D5	10 months	14	male	HC5	27	female	HR5	18	male	Placebo	no	
T1D6	1 month	11	male	HC6	23	female	HR6	11	male	Placebo	no	
T1D7	3 months	13	female	HC7	25	female	HR7	48	male	Nicotinamide	no	
T1D8	20 days	9	female	HC8	25	male						
T1D9	1 month	5	male	HC9	31	female						
				HC10	24	male						

Laboratory methods

Isolation of peripheral blood mononuclear cells (PBMC)

For all papers included in this thesis; peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque density gradient centrifugation (Pharmacia Biotech, Sollentuna, Sweden) from sodium heparinised venous blood samples. Sodium heparinised blood samples were initially diluted 1:2 in room tempered RPMI 1640 medium (Gibco, Täby, Sweden) and then gently transferred onto Ficoll and thereafter centrifuged for 30 minutes at 400g. The erythrocytes at this stage are localised to the bottom of the tube, while the smaller, lighter, mononuclear cells are collected from the interphase of Ficoll and the overlaying fluid. Following this step, PBMCs were washed twice, by 15 minutes of centrifugation at 400g, in RPMI 1640 supplemented with 2% foetal calf serum (FCS, Gibco). Cell numbers were counted using light microscopy, following staining with Türks solution (0.04g crystal violet to 50g 25% acetic acid and 150 ml MilliQ H_20) at a concentration of 1:10. PBMCs were then washed a third and final time.

Since blood samples were taken on different occasions, PBMCs were cryo-preserved in liquid nitrogen for batch analysis. Freezing medium (40% RPMI 1640, 10% DMSO

(dimethyl sulfoxide, Sigma, Stockholm, Sweden) and 50% FCS) was added drop-wise to PBMCs resulting in a cell suspension of $5x10^6$ cells/ml, put into aliquots in cryotubes and freezed at -70ºC in a pre-cooled (4ºC) freezing container (Mr Frosty NALGENE Labware, Rochester, USA), allowing a lowering of the temperature of 1ºC/minute. The following day the cryotubes were transferred into liquid nitrogen for storage until further use.

At time of use, PBMCs were thawed directly in a 37ºC water bath under continuous agitation and immediately washed once in RPMI 1640 supplemented with 10% FCS, and thereafter resuspended in AIM V research grade serum free medium (Gibco) with supplements (2mM L-glutamine, 50µg/L streptomycin sulphate, 10µg/L gentamicin sulphate and $2x10^{-5}$M 2-mercaptoethanol). After counting in Türks solution, the membrane integrity was determined by Trypan blue (0.2g Trypan blue and 0.9g NaCl in 100ml MilliQ H_20) exclusion.

Cell culturing

For papers I and III aliquots of 1.5×10^6 PBMCs (viability approximately 90% or more for each population) were diluted in 1500µl AIM V research-grade serum-free medium (Gibco) supplemented with 2 mM L-glutamine, 50µg/l streptomycin sulphate, 10µg/l gentamicin sulphate and 2×10^{-5} M 2-mercaptoethanol (Sigma). PBMCs were incubated in medium alone (spontaneous expression/secretion) or with GAD_{65} protein (Diamyd™, Diamyd Therapeutics AB, Stockholm, Sweden) and phytohaemagglutinin (PHA, Sigma) at a concentration of 5 µg/ml [136-138] at 37ºC in a humidified atmosphere with 5% CO_2. In samples with a limited number of cells, the order of priority for stimulation was spontaneous *in vitro* culture, PHA and finally GAD_{65}. Cell-supernatants were harvested after 48 hours *in vitro* for further detection of cytokines and chemokines by protein microarray (paper I) and study of messenger-ribonucleic acid (mRNA) expression by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and soluble CTLA-4 by Gyros technology (paper III).

In paper II, the same principal methodology with the following alterations was used; PBMCs were incubated with medium alone (spontaneous expression/secretion) or

stimulated with 5μg/ml [138] of the autoantigens GAD_{65} (protein, Diamyd Diagnostics AB) and the synthetic peptide of GAD_{65} a.a. 247-279 (NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSE-OH) molecular weight 3823.7 (Dept of Medical and Physiological Chemistry, University of Uppsala, Sweden) and a peptide of heat shock protein 60 (HSP_{60}) a.a. 437-460 (DiaPep277, a gift from B.Roep, Leiden University Medical Center, Leiden, the Netherlands) and the mitogen PHA (Sigma) (order of priority in case of inadequate cell count; spontaneous, GAD_{65}-protein, HSP_{60}-peptide, GAD_{65}-peptide, and finally PHA) at 37˚C, in a humidified atmosphere with 5 % CO_2. PBMCs were harvested after 72 hours of *in vitro* culture for analysis of mRNA expression and cell culture supernatants were saved for analysis of cytokines and chemokines.

Protein microarray

In paper I, cytokines and chemokines were detected by protein microarray, performed with a commercially available kit according the manufacturer´s instructions (kit no H0108001, RayBiotech, Norcross, GA, USA). Cytokines have traditionally been detected by ELISA; however the protein microarray offers a more sensitive method with less variation, as well as the concurrent detection of several cytokines

Medium harvested from spontaneous, GAD_{65} protein and PHA stimulated, 48 hour *in vitro* cultures (see section *Cell culturing*) were used for detection. Each membrane was incubated with 2 ml 1X Blocking Buffer and incubated at room temperature for 30 minutes. Blocking Buffer was decanted from the membrane containing vessel and the membrane incubated with sample (culture medium) at room temperature for 1 - 2 hours. The sample was thereafter drained from each vessel, and membranes washed three times with 2 ml 1X Wash Buffer I for five minutes at room temperature with agitation. Another round of washing was commenced twice, with 2 ml of 1X Wash Buffer II, for five minutes at room temperature with agitation. A working stock of primary antibodies was prepared by adding 4 μl of biotin-conjugated antibodies to 996 μl of 1X Blocking Buffer, and then added to each membrane. Antibody drenched membranes were then incubated at room temperature for 1-2 hours, followed by the two washing steps described above. Following this step, each membrane was incubated with 2 μl HRP-conjugated streptavidin to 1998 μl 1X Blocking Buffer, for

30-60 minutes at room temperature, followed by yet another round of washing before the detection reaction was commenced. Before detection, the membrane was incubated at room temperature in a mixture of Detection Buffer C and D (1:2), for five minutes and then drained of excess reagent.

Detection was achieved using the Fuji LAS1000 imaging system and chemiluminescent signals were analysed using the AIDA software (Advanced Image Data Analyzer, 3.28, Raytest Izotopenmessgeraete, Straubenhardt, Germany). The intensity of spots (%) was calculated. The sensitivity was as follows: IL-5, 1 pg/ml; IL-6, 1 pg/ml; IL-7, 100 pg/ml; IL-10, 10 pg/ml; TNF-α, 100 pg/ml; TNF-β, 1000 pg/ml; TGF-β, 200 pg/ml; IFN-γ, 100 pg/ml; MCP-1, 3 pg/ml and for MIG, 1 pg/ml (RayBiotech) (Fig. 6).

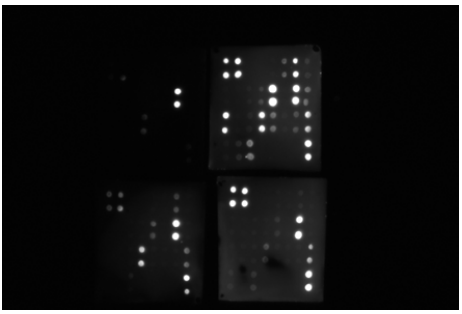

Figure 6 *Signal intensity of bound cytokine/chemokine-antibody complexes.*

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Real-time RT-PCR was used in paper II and III and is a widely used and sensitive technique in molecular biology to simultaneously amplify and quantify targeted mRNA molecules. Messenger RNA to be copied is first transcribed into complementary DNA (cDNA) by a polymerase with reverse transcriptase activity. This is the "RT" part of the RT-PCR. Generated cDNA is later amplified by regular PCR. Real-time RT-PCR is best suited for quantitative information.

We used a multiplex PCR technique where FAM dye is used for the quantification of the target mRNA simultaneously with VIC dye quantification of an endogenous control (18S ribosomal RNA) in the same well. As the 18S is a housekeeping gene expressed with low variation, the simultaneous detection of target and control allows

for comparison of quantitative target expression over the sample set being studied. We also used a passive reference that contained the dye ROX, which allows compensation for variations in fluorescence between wells. These types of variations can be due to the design of the instrument or pipetting errors. Due to the constant concentration of ROX during PCR, the fluorescence of the dye is unwavering. In this way, ROX offers a stable baseline for sample normalisation for non-PCR-related fluctuations in the fluorescence.

RNA isolation and cDNA synthesis

Total RNA was isolated from PBMCs with RNeasy 96 as recommended by the supplier (Qiagen, KEBO, Spånga, Sweden) and quantified by optical density (OD) at 260nm. Using equal amounts of total RNA (7 ng/μl) from PBMCs stimulated under various conditions, mRNA was marked complementary with random hexamers, and complementary DNA (cDNA) was synthesised from the mRNA using a High Capacity cDNA archive kit (Applied Biosystems, Stockholm, Sweden), according to the manufacturer´s description. In addition to the manufacturer´s instructions, 1 U/ml of RNAse inhibitor (Applied Biosystems) was added to the reaction.

Multiplex RT-PCR

In paper II and III markers associated with Tregs (FOXP3, CTLA-4, and TGF-β) were detected by multiplex real-time RT-PCR, as described previously [139]. The contents of the PCR-reaction mixture included TaqMan Universal PCR Master Mix (Applied Biosystems), specific target primers and probe (CTLA-4: Hs00175480, FOXP3: Hs00203958 and TGF-β: Hs00171257, all FAM dye layer, Applied Biosystems) and endogenous reference primers and probe (18S rRNA: 4310893E, VIC dye layer), for normalization of each individual sample to the housekeeping gene 18S.

For soluble CTLA-4, in paper III, custom-designed primers and probe (described previously [139]) were used under the same reaction conditions. Primers and probe were designed using Primer3 (*http://frodo.wi.mit.edu/primer3/*) and tested in BLAST with no alternative hits. To avoid binding to full length CTLA-4, the primers/probe for the soluble CTLA-4 assay were designed so that one half was complementary to mRNA encoding the extracellular domain and the other half to mRNA encoding the

intracellular domain. The transmembrane domain of the full length CTLA-4 transcript interrupts this sequence and prevents the faulty detection of full length CTLA-4 as soluble CTLA-4. Sequences are as follows: soluble CTLA-4 forward primer = 5'-CATCTGCAAGGTGGAGCTCAT, reverse primer = 5'-GGCTTCTTTTCTTTAGCAATTACATAAACT and probe = 5'-6-FAM-ACCGCCATACTACCTGGGCATAGGCA-TAMRA. The reaction mixture was amplified using the 7500 Fast Real-Time PCR system (Applied Biosystems) for 50 cycles with an annealing temperature of 60°C. The FAM dye layer yields the results for quantification of the target mRNA while the VIC dye layer yields the results for quantification of the 18S ribosomal RNA endogenous control. The passive reference contained the dye ROX in order to normalise for non-PCR-related fluctuations in the fluorescence signal. In all experiments, samples were loaded in duplicate and both non-template controls and the in-house-control (unstimulated PBMCs of a healthy individual) were included in the duplicates.

Calculation of relative quantification values

The relative quantification values for the mRNA gene expression assays were calculated from the accurate threshold cycle (CT), according to the manufacturer's description (protocol P/N 4304671, Perkin Elmer). The accurate CT represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, obtained from both dye layers in the assay. This means that a low CT value represents a high mRNA expression and vice versa. The CT value for rRNA (VIC) was subtracted from the CT value of the immunological marker (FAM) to calculate the Δ-CT for the calibrator and samples in each Treg associated marker gene expression assay. The Δ-CT values for duplicate wells for each immunological marker were averaged. Values were mathematically recalculated as quotas of the averaged Δ-CT values ((1/averaged Δ-CT)*100) to represent a high expression by a high number and vice versa.

Gyros

In paper III, soluble CTLA-4 was detected using an in-house sandwich immunoassay developed for the Gyrolab Bioaffy platform (Gyros AB, Uppsala, Sweden) [140]. As capture antibody, a biotinylated anti-human CD152 (AS33-B, Antibody Solutions, Palo Alto, CA) followed by complementary anti-human CD152 antibody (BNI.3, BD PharMingen, San Diego, CA) previously labelled with Alexa Flour 647 (Molecular

Probes, Eugene, OR, USA) were used, and have previously been shown suitable for sandwich immunoassay [101].

Generally, the Gyros technology is based on a CD microlaboratory, Gyrolab Bioaffy, containing 104 identical microstructures, connected in groups of 8, to generate 104 data points (Fig. 7). Each microstructure contains a 15 nl streptavidin column to which biotinylated capture antibodies can be attached. The CD microlaboratory is inserted into a working station that will perform all transfers of liquid from a microtiter plate, where all reagents and samples have been prepared, into an integrated fluorescence detector for laser-induced fluorescence (LIF). The liquid transfer is performed by capillary force and further movement of liquids within the CD microlaboratory is driven by centrifugal force when spinning the CD. Samples and detection antibodies, previously labeled with Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA), will be added, following the attachment of capture antibodies to the streptavidin columns, to form a sandwich immunoassay. The detected fluorescence is compared with a standard curve processed in an identical fashion.

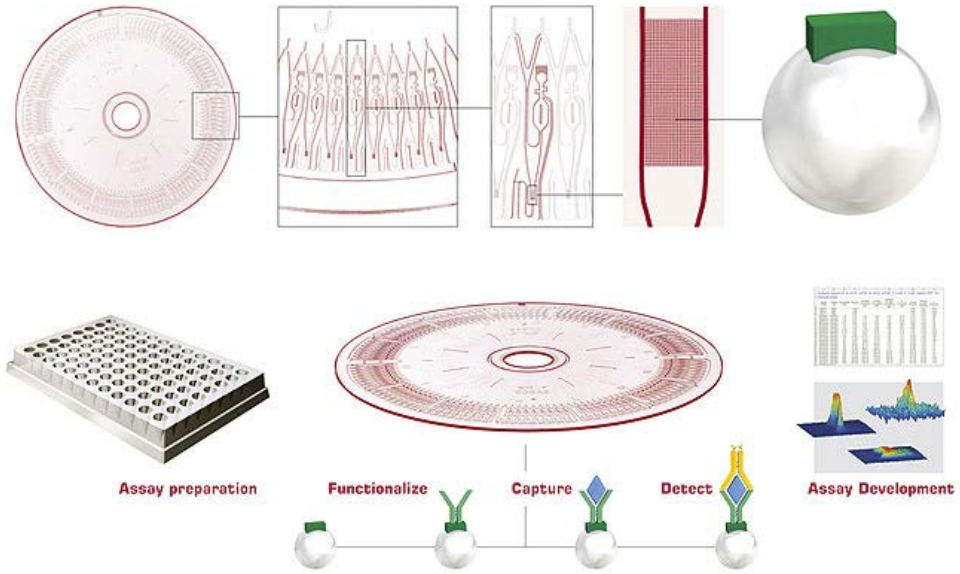

Figure 7 *The top of the figure displays the principal construction of the CD microlaboratory. Samples and reagents are prepared in a microtiter plate and transferred to the CD microlaboratory by capillary force and distributed in the microstructures by centrifugal force. Results is gained as fluorescence and re-calculated to concentrations. (Gyros AB)*

Culture supernatants and serum samples were slowly thawed on ice. The samples were then vortexed and centrifuged for 15 minutes at 4000 rpm and 8°C. Each sample was then diluted 1:10 (1 μl + 9 μl buffer) in microtiter plates in duplicate. Capture- and later, complementary antibodies, as well as samples, were added to the microlaboratory and after each addition of reagents or samples to the microstructure, the streptavidin columns were washed repeatedly using 0.01 M phosphate buffered saline, pH 7.2, containing 0.01% Tween-20 (PBS-T).

An eight point standard curve (10–0.0015 ng/ml range), also including a blank, was generated with the CTLA4-Ig protein (Ancell, Bayport, MN). Soluble CTLA-4 protein was quantified in duplicate and concentration calculated using Gyrolab Evaluator software (Gyros AB, Uppsala Sweden). The sensitivity limit for this assay was approximately 0.062 ng/ml.

Luminex

The multiplex fluorochrome technique, Luminex™, is a bead-based sandwich immunoassay that combines the enzyme-linked immunosorbent assay (ELISA) and flow cytometry techniques. The technique employs monoclonal antibodies directed against the cyto- and chemokines of interest, bound to beads with a known internal fluorescence, and allows simultaneous detection of up to 96 cyto-and chemokines in a dual laser flow analyser (Luminex 100™, xMAP™ technology, Luminex Corp, Austin, Texas, USA).

In paper II, cytokines (IL-1β, -6, -7, -10, -13, -17, IFN-γ and TNF-α) and chemokines (IP-10, MCP-1, RANTES, MIP-1α and MIP-1β) associated with Th1, Th2, Tr1 and other cells involved in inflammation, were detected in the cell supernatants by multiplex fluorochrome technique (Luminex™). The assays were performed using Bio-Plex™ Human Cytokine 7-plex Panel (IL-1β, -6, -7, -10, -13, IFN-γ and TNF-α), Bio-Plex™ Human Cytokine single-plex Panel (IL-17) and Bio-Plex™ Human Cytokine 5-plex Panel (IP-10, MCP-1, RANTES, MIP-1α and MIP-1β) (Bio-Rad Laboratories, CA, USA), according to the manufacturer's instructions. In all assays, the Bio-Plex™ Cytokine Reagent Kit (Bio-Rad Laboratories) was used.

Cell culture supernatant (50 μl) in single or standard in duplicate along with 50 μl of beads were added to the wells of a pre-wet 96-well microtiter plate. After a 30 minute incubation with agitation, the plate was washed and 25μl of biotinylated detection antibody mixture was then added and the plate again incubated for 30 minutes. After washing, 50 μl of streptavidine-PE was added and the plate incubated for 10 minutes before a final wash and resuspension of beads in 125μl assay buffer. Identification and quantification of each bead subset was accomplished using a Luminex 100™ instrument (xMAP™ technology, Luminex Corp, Austin, Texas, USA). A minimum of 100 beads per region were analysed.

Raw data was analyzed using Starstation Software version 2.3 (Applied Cytometry Systems, Sheffield, UK) and a curve fit was applied to each standard curve according to the manufacturer's instruction. Values of sample concentrations were interpolated from the standard curves. The cut-offs for minimum detectable concentrations were as follows: IL-1β (0.6 pg/ml), -6 (0.37 pg/ml), -7 (0.74 pg/ml), -10 (0.35 pg/ml), -13 (0.07 pg/ml), -17 (0.66 pg/ml), IFN-γ (0.40 pg/ml), TNF-α (1.43 pg/ml), IP-10 (0.82), MCP-1 (0.43 pg/ml), RANTES (0.42 pg/ml), MIP-1α (0.17 pg/ml) and MIP-1β (0.38 pg/ml).

Flow cytometry/cell sorting

To be able to study Tregs, starting with small sample sizes due to restricted sampling from T1D children, one goal of the study presented in paper IV was to gain a significant expansion of Treg numbers. At times, the only logical option when working with patient material is to cryopreserve PBMCs. Cryopreservation may in part further restrict the amount of cells available, which is why efficient methods are of outmost importance. Hence, in paper IV we sought to investigate the cryostability of Treg associated markers and subsequently sort and expand Tregs from cryopreserved PBMCs of T1D, high-risk and healthy individuals. The aim was to efficiently expand Tregs and to detect any difference in T-cell number and composition among the studied subjects.

As described previously, the characterisation and detection of Treg cells is not an easy task, due to the lack of a truly unique Treg marker and the increasing bulk knowledge of a cell type more plastic than we previously imagined. However, a lot

of attention has been paid to try and characterise a combination of markers to identify these suppressive T cell subsets. While the so called classic Treg gating strategy, based on the concurrent expression of CD4 and the highest expression of CD25 (Fig. 8), comprises only about 1-2% of the $CD4^+$ cells, we did not find it optimal for sorting, when starting with a limited sample size. However, Liu *et al* [73] demonstrated that Tregs defined by the concurrent expression of $CD4^+CD25^+CD127^{lo/-}$, as gated in figure 9, comprised a larger cell number but were as suppressive. Hence, we decided to use this strategy.

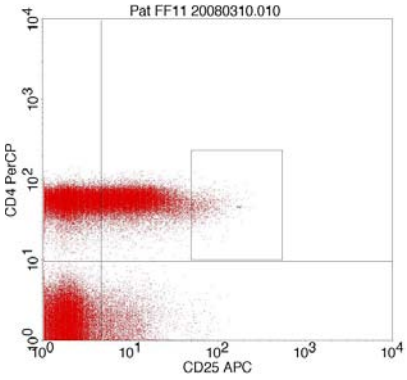

Figure 8 *Treg gating based on high CD25 expression in CD4 positive lymphocytes*

Antibodies

For staining and sorting, fluorescein isothiocyanate (FITC)-conjugated anti-CD127, allophycocyanin (APC)-conjugated anti-CD25 (both from eBioscience, San Diego, CA, US) and pacific blue (PB)-conjugated anti-CD4 (clone OKT4, produced in-house) and Alexa 700-conjugated anti-FOXP3 (eBioscience) mAbs, were used. For comparison of marker expression in the pre-study, FITC-conjugated anti-FOXP3 (Nordic BioSite, Stockholm, Sweden), APC-conjugated anti-CD25 and peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD4 (Becton Dickinson (BD) Biosciences, San Jose, USA) mAbs were used. PBMCs were isolated (as described in section "Isolation of peripheral blood mononuclear cells (PBMC)") and stained either fresh (for the pre-study) or post thaw, for 30 minutes at 4°C and washed in phosphate buffered saline (PBS) supplemented with 2% human serum. For intracellular staining, Alexa 700-conjugated anti-FOXP3 was used following fixation and permeabilization with appropriate buffers (Miltenyi Biotech, Paris, France).

Flow cytometry acquisition and sorting

PBMCs were analyzed and sorted using a FACSAria (Becton Dickinson) equipped with 488, 633 and 407 nm lasers. Lymphocytes were gated based on forward (FSC) and side scatter (SSC). For examination of Treg-marker expression before and after cryopreservation, in the pre-study, $CD25^{hi}$ cells were gated as $CD4^+$ T lymphocyte subsets expressing higher levels of CD25 than the discrete population of CD4- cells expressing CD25 (Fig. 8). FOXP3 expression was then analysed in this gate. Also for sorting, CD4 expressing lymphocytes were gated to further obtain a dot plot of CD25 and CD127 fluorescence. Tregs were gated as $CD4^+CD25^+CD127^{lo/-}$ and compared to $CD4^+CD25^-$ and $CD4^+CD25^+CD127^+$ subpopulations (Fig. 9). The concurrent expression of CD25 and FOXP3, following expansion of $CD4+CD25^+CD127^{lo/-}$ and $CD4^+CD25^-$ cultures, were analysed and compared to each other. The cut-offs for the gates were set after the fluorescence of a biologically FOXP3 negative and CD25 negative population. Data was analysed using the FlowJo software (Tree Star, Inc., Ashland, Oregon) and expressed as mean fluorescence intensity (MFI; geometrical and standard mean) and percentages of cells expressing each marker.

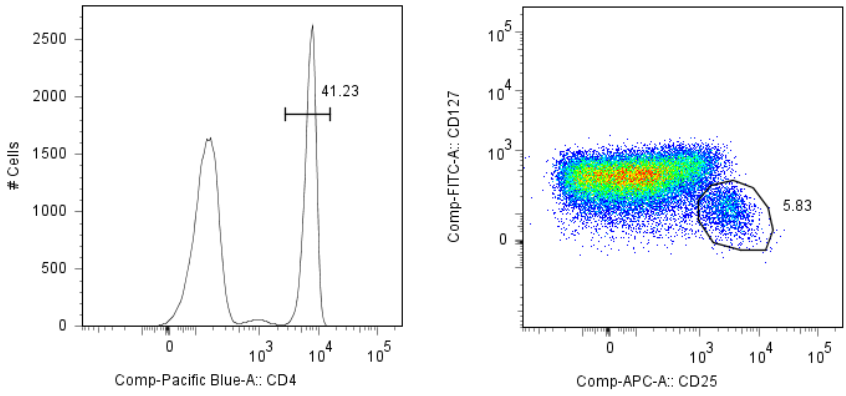

Figure 9 *Gating strategy for sorting of $CD4^+CD25^+CD127^{lo/-}$ Tregs. Lymphocyte population was gated based on forward and side scatter and from this gate $CD4^+$ cells were isolated and further gated for expression or no expression of CD25 and CD127.*

In vitro expansion

Tregs were expanded according to a protocol adapted from Putnam et al. [141]. Briefly, on day 0 sorted cells were resuspended in AIM-V medium (Gibco/Invitrogen) containing 10% HS and amphotericin B and plated according to table 2. Dynabeads® Human Treg Expander anti-CD3/anti-CD28 coated microbeads

(Invitrogen; catalogue number 111.61D) were added at a 1:1 bead to cell ratio. When Treg numbers were lower than 40.000, 96-well flat-bottomed plates were used. The cell culture volume was doubled at day 2 and IL-2 (Proleukin, Chiron Therapeutics, Emeryville, CA) added at a final concentration of 300 U/ml. On days 5 and 7, cells were counted, washed in AIM-V and resuspended as above, adding fresh IL-2. Restimulation with anti-CD3/anti-CD28 coated microbeads, were performed on day 9, as described for day 0. Cells were counted again on day 11 and 13, washed, resuspended according to table 5, and supplemented with fresh IL-2. Cultures were terminated on day 15 and cells stained for FOXP3 analysis.

Table 5 Description of the experimental setup for expansion of CD4+CD25+CD127lo/-. x2=equal volumes were added at day 0 and again at day 2. For culture split or re-stimulation, the double volume was added.

Expansion of Treg				
Cell Number	**Vessel**	**Media Volume**	**Dyanabeads**	**rhIL-2**
40 000	96 well	125µl x2	1µl	300U/ml
100 000-150 000	48 well	500µl x2	2.5-3.75µl	300U/ml
200 000-300 000	24 well	1ml x2	5-7.5µl	300U/ml
400 000	12 well	1.5ml x2	10µl	300U/ml
800 000	6 well	2ml x2	20µl	300U/ml
1 200 000	Vertical T25	3ml x2	30µl	300U/ml
2 400 000	Horizontal T25	5ml x2	60µl	300U/ml
7 500 000	T75	15ml x2	187.5µl	300U/ml
17 000 000	T175	35ml x2	425µl	300U/ml

CD4+CD25- cells were expanded according to a scheme similar to Tregs, with the following alterations. As anti-CD3/anti-CD28 coated microbeads caused overstimulation and activation-induced apoptosis, CD4+CD25- cells were expanded using anti-CD3 (OKT3, 10µg/ml) coated culturing vessels (Table 6) and soluble anti-CD28 (1µg/ml). IL-2 addition was added at a concentration of 30 U/ml.

Table 6 Description of the experimental setup for expansion of CD4+CD25- cells. x2=equal volumes were added at day 0 and again at day 2. For culture split or restimulation, the double volume was added. Vessels were precoated with antiCD-3 (OKT3 10 µg/ml)

Expansion of Teff				
Cell Number	**Vessel**	**Media Volume**	**CD28**	**rhIL-2**
<500 000	96 well	125µl x2	1µg/ml	30U/ml
500 000	48 well	500µl x2	1µg/ml	30U/ml
$1x10^6$	24 well	1ml x2	1µg/ml	30U/ml
when cells exceed 2.5x106 or medium turns yellow. split back to 1x106 /24 well				
$50x10^6$	T175	40mlx2	1µg/ml	30U/ml

C-peptide

A time-resolved flouroimmunoassay (AutoDELFIA C-peptide kit, PerkinElmer,Wallac, Turku, Finland) was used to measure C-peptide levels in serum samples of fasting high-risk individuals and T1D children. Validation of assays was performed by including a C-peptide control module containing a high-level control as well as a medium-level and low-level control (Immulite, Siemens Healthcare Diagnostics Products Ltd, Llanberis, Gwynedd, UK). Automatic measurement and calculation were carried out using the software 1224 MultiCalc, Wallac, and results were expressed in nanomoles per litre.

HLA-genotyping

For healthy- and T1D children, HLA genotyping for DQB1 and DQA1 alleles was performed using an oligonucleotide hybridization assay, as described in detail elsewhere [142-144]. High-risk individuals were genotyped for HLA genotype during the ENDIT-trial as described elsewhere [145].

Statistics

In the projects included in this thesis, several statistical methods have been applied. As we observed no Gaussian distribution of the data (even after logarithmic transformation), non-parametric methods were used. Two groups were compared by Mann-Whitney U-test, and three or more groups using the Kruskal-Wallis test for unpaired observations. In paper I, Spearman's rank correlation was used when comparing paired non-parametric variables. For pair-wise comparisons, Wilcoxon signed rank test was used. A probability level of $p<0.05$ was considered to be statistically significant.

Calculations were performed using the following statistical packages; paper I and III StatView 5.0.1 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA), paper II and IV GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., San Diego, CA, USA),

Ethical considerations

Blood samples included in this study was drawn after informed consent was obtained from all study subjects above 18 years of age, or the responsible guardians. The studies included in this thesis were approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University, Linköping, Sweden.

RESULTS AND DISCUSSION

We have observed a markedly altered immune profile in individuals at high risk of developing T1D, years prior to being clinically defined as having the disease.

Th1-associated immune profile

Possible elevation in high-risk individuals (pre-diabetic phase)

Type 1 diabetes has long been considered to be a Th1-associated disease. A burst of IFN-γ, that exceeded the secretion in healthy children but interestingly also that observed in children at the onset of T1D [69], In accordance with this description, we observed elevated levels of IFN-γ and TNF-β, by protein microarray, in individuals considered to have as high as a 40% chance of developing T1D within 5 years, both spontaneously (IFN-γ) and following stimulation with the autoantigen GAD_{65} (Fig. 10).

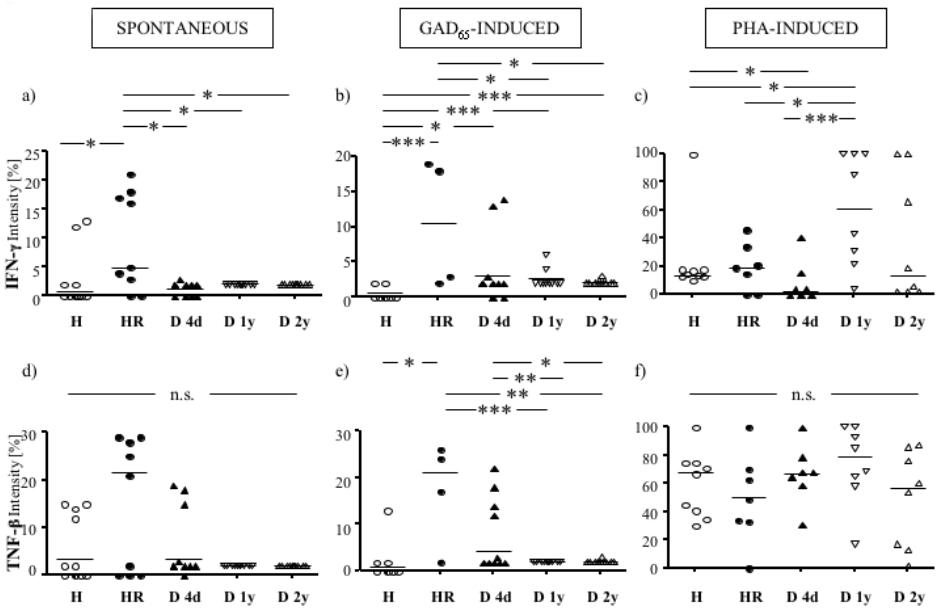

Figure 10 *The Th1-associated cytokines IFN-γ and TNF-β secreted spontaneously (a, d) or from stimulation with either GAD_{65} (b, e) or PHA (c, f) presented as intensity (%).H = healthy, HR = high-risk, D 4d = T1D children four days after diagnosis, D 1y = T1D children one year after diagnosis, D 2y = T1D children two years after diagnosis. * p<0.05, ** p=0.01, *** p<0.01, n.s. = not significant*

Taken together, the prodrome leading up to clinical onset of T1D, rather than the clinical diagnosis itself, seems to be accompanied by an elevation of Th1-activity. Considering that T1D is caused by a defective resolution of a local pancreatic inflammation (insulitis) which leads to autoimmune destruction of β-cells, it is not surprising to find signs of these immunological processes already before clinical symptoms appear. What is interesting is that we see alterations from the immunological profile of healthy children, in the peripheral blood, already years before the high-risk individuals progressed to T1D. A previous study displayed high levels of IFN-γ, 2-5 years before T1D onset and also indicated that these levels decreased towards onset of disease [146]. If we can learn to recognize the immunological features of the individuals that will progress to have the disease, there might be a possibility to counteract the inflammatory process and postpone the diagnosis, or even prevent it.

Suppression following clinical onset of T1D

As mentioned above, the pre-diabetic phase has been seen to feature an elevated Th1-activity (*e.g.* IFN-γ, TNF-β), probably reflecting the untamed immunological process responsible for the β-cell destruction in the pancreas. We find these elevated secretions to be decreased or suppressed at disease onset and staying low for up to two years following diagnosis (Fig. 10). This is in agreement with previous reporting of a decrease of the Th1-like dominance of the pre-diabetic phase, following the diagnosis of T1D [137, 146-148]. Following GAD_{65} stimulation however, there is still an increased secretion of IFN-γ when compared to healthy children, even though it is lower than the secretion induced from high-risk individuals (Fig. 10b). This observation is further supported from studies implicating reduced spontaneous IFN-γ secretion and the Th1-associated chemokine receptor CCR5 mRNA expression, reduced mitogen-induced IFN-γ secretion as well as significantly lower percentages of peripheral IFN-γ producing CD4+ and CD8+ cells in recent-onset T1D patients [70, 149-152].

In contrast to our previous studies [147, 153] we were not able to show these differences regarding IFN-γ secretion, neither spontaneously, nor following autoantigen or mitogen stimulation with multiplex technology (Luminex) in paper II. We did, however, find that spontaneous IP-10 secretion was lower at clinical T1D

onset from that seen at high-risk, and also that both newly diagnosed T1D and high-risk individuals had a lower specific secretion following stimulation with GAD_{65} compared to the response seen from healthy children (Fig. 11a-b). On the contrary, high-risk individuals had a lower response to mitogen stimulation (Fig. 11c). IP-10 is a chemokine secreted by a variety of cell types in response to IFN-γ. Whether there actually are no differences present for IFN-γ in this study cohort, or the method is not sensitive enough, is hard to determine. However, the lowered IP-10 secretions in newly diagnosed T1D children follow our previous findings of a decreased IFN-γ response at diagnosis of T1D [137, 153, 154].

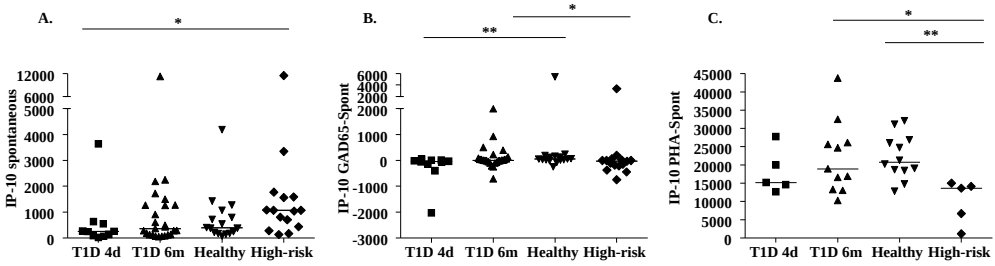

Figure 11 *Spontaneous, GAD_{65} and PHA stimulated secretions of interferon-inducible protein (IP)-10, measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test for two groups, and three or more groups using the Kruskal-Wallis test for unpaired observations. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01*

One can speculate as to why there is a difference between these observations. For example, cells were harvested at different time points (72hrs in paper II and 48hrs in paper I. A previous study investigated the optimal time points for cytokine profile assessment in whole blood of healthy female donors. They found that intracellular levels of IFN-γ, following PHA- and cytolomegavirus-stimulation, peaked around day three and likewise for extracellular levels. The difference observed was that the latter remained relatively stable between days three and seven, while intracellular levels decreased steadily from day three [155]. Taking this into account, it might not be unreasonable to ponder the possibility that IFN-γ at 72hrs of stimulation will have reached a maximum point and as such could hide possible differences between the study groups. Another explanation could be attributed to different methodologies employed. Even though the multiplex technology detects at very low concentrations, the protein microarray used in paper I might be more stable.

Th2-associated immune profile

When studying the Th2-associated profile (*i.e.* IL-13 and IL-5), we found spontaneous secretion of IL-13 to be suppressed in both high-risk individuals and T1D (Fig. 12a). Not surprisingly, high-risk individuals exhibited a lower spontaneous quota of IL-13 to IFN-γ in comparison to both healthy and newly diagnosed T1D children, as well spontaneously as induced by GAD_{65} (protein and peptide) and HSP_{60} (Fig. 12c-f). Specific secretions to autoantigens further presented decreased quotas IL-13 to IFN-γ from new-onset to six months post diagnosis. The mitogen induced secretion of IL-13 and IL-5 showed unanimous results. While we saw that high-risk individuals responded to mitogen-stimulation with a high IL-5 secretion, much higher than seen in T1D, even though the secretion in T1D also showed an elevation in comparison to healthy children (Fig. 13), we also saw a lower mitogen induced IL-13 secretion, as in comparison to T1D children at six months post diagnosis (Fig. 12b).

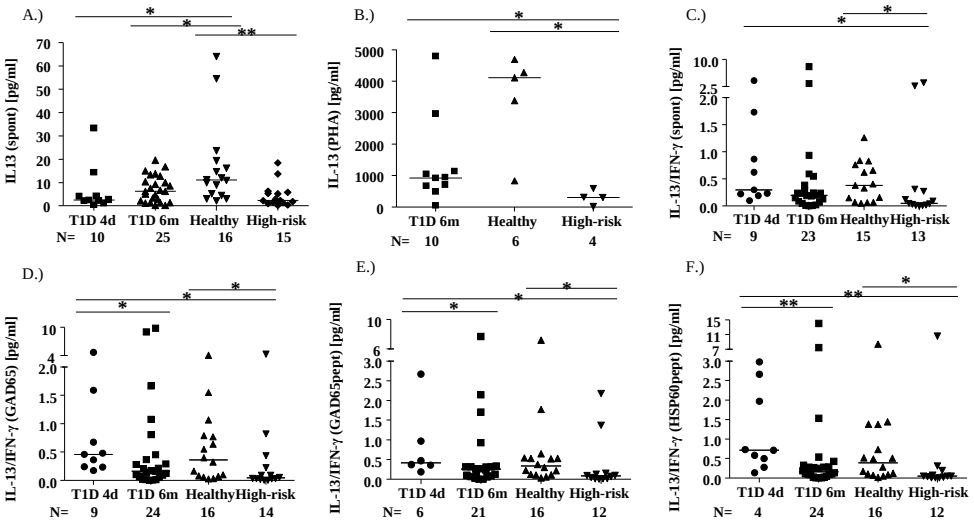

Figure 12 *Spontaneous and phytohaemagglutinin (PHA) stimulated secretions of interleukin (IL)-13 (a-b). The quota of IL-13 to interferon (IFN)-γ spontaneously (c) and following stimulation with GAD_{65}-protein (d), the GAD_{65}-peptide (a.a. 247-279) (e) and the heat shock protein $(HSP)_{60}$-peptide (a.a. 437-460) (f). Secretions measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test for two groups and Kruskal-Wallis test for unpaired observations, for three or more groups. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01*

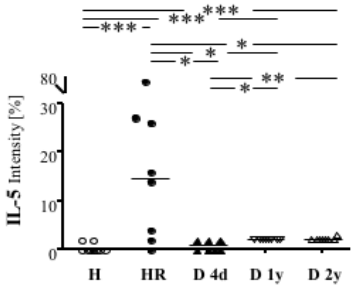

Figure 13 *PHA-induced secretion of the Th2-associated cytokine IL-5 presented as intensity (%). H, healthy; HR, high-risk; D 4d, T1D children 4 days after diagnosis; D 1y, T1D children 1 year after diagnosis; D 2y, T1D children 2 years after diagnosis; PHA, phytohaemagglutinin; Th, T-helper; IL, interleukin; T1D, type 1 diabetes. *p < 0.05, **p = 0.01, ***p < 0.01*

One could speculate that a decreased IL-13 secretion might contribute to a low ability to keep a significant Th1-like activation in check, as IL-13 is thought to antagonize Th1 responses [56, 156]. Deepak *et al* showed that an early antigen-specific Th2-induction to the putative autoantigens GAD_{65} and HSP_{60} increased the population of cells secreting IL-13, which in turn suppressed Th1-like immune responses [56]. It is appealing to speculate that an induction of Th2 immunity, specific to the major autoantigens in T1D, could be beneficial in the pre-diabetic phase of high-risk individuals, helping to stem the strong Th1 activation associated to the attack on the pancreatic islets and thereby maintaining residual C-peptide. However, previous studies have intended to restore tolerance by administration of the target antigens, but this far with modest success. Neither oral nor injected insulin were shown to prevent or delay T1D in the Diabetes Prevention Trial of Type 1 Diabetes (DPT-1), except for when a subgroup positive for insulin autoantibodies (IAA) were analysed [157]. Neither did nasal insulin prevent or delay T1D, in The Type 1 Diabetes Prediction and Prevention Study (DIPP) using Finnish newborns carrying high-risk genotypes [158]. Treatment with the 437-460 peptide HSP_{60}, p277 (DiaPep277) have reported alterations from Th1 to Th2 immunity and also induction of IL-10 [159, 160]. Nonetheless, the effects on β-cell preservation are presently only seen in adults and thus far, modestly [161]. Despite promising data from animal studies, neither the GAD_{65} vaccination phase IIa and b trials (Diamyd Medical) showed any greater success for the patients, even if immunological changes and, in subgroups, a better preservation of the C-peptide occurred [162, 163]. It is probable that high-risk individuals need to be identified early, clearly defined based on diabetes related genetics and autoantigens, and before the autoimmune attack on the β-cells have escalated. This criteria should be able to prompt a specific and suppressive Th2-like milieu; perhaps through antigen based therapy.

Pro-inflammatory activity

Nicotinamide treatment

As some of the investigated high-risk individuals had previously received nicotinamide treatment in the ENDIT study, we wanted to see whether there were any differences between those individuals and the ones that received a placebo. We found the pro-inflammatory cytokines IL-6 and MIP-1α, secreted spontaneously, to be lower in the high-risk individuals receiving nicotinamide as compared to those receiving a placebo (Fig. 14a-b). Even though nicotinamide did not prevent T1D in the ENDIT study [135], it suppressed inflammatory mediators, confirming previous suggestions of a moderately altered immune response in those high-risk individuals treated with nicotinamide [146, 164].

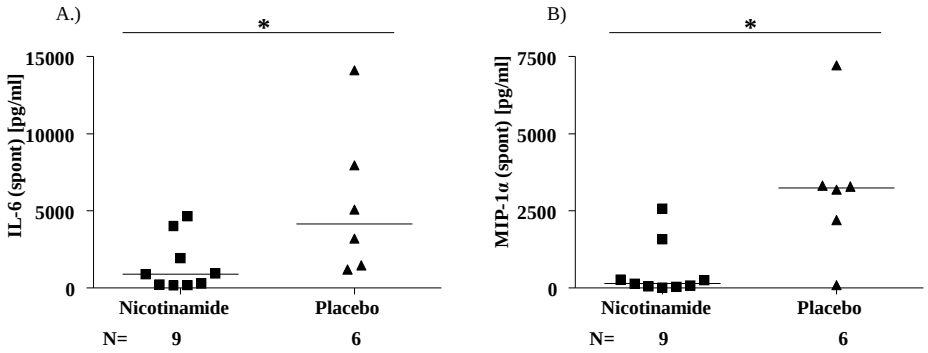

Figure 14 *Spontaneous secretion of interleukin (IL)-6 (a) and Machrophage Inflammatory Protein (MIP)-1α (b) in high-risk individuals that had received nicotinamide treatment in comparison to those that received placebo. Secretions measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test. The bars in each figure correspond to the median values.*
**=p<0.05*

Clinical onset of T1D

In contrast to the suppressed Th-1 activity at clinical onset, we found an increase of pro-inflammatory cytokines (TNF-α and IL-6, Fig. 15) and chemokines (MCP-1 and MIG, Fig. 16) from the pre-diabetic stage to clinical onset of T1D in paper I, both spontaneously, as well as following GAD_{65} stimulation. MIG is induced by IFN-γ in human islets [165], explaining the positive correlation observed between MIG and IFN-γ in our cohort.

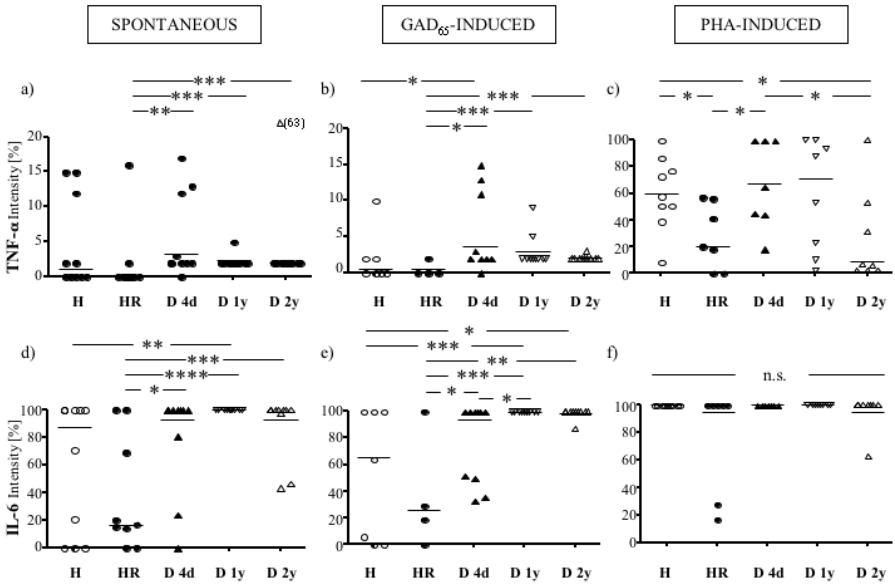

Figure 15 *The pro-inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-6 secreted spontaneously (a, d) or from stimulation with either GAD_{65} (b, e) or PHA (c, f) presented as intensity (%). H, healthy; HR, high-risk; D 4d, T1D children 4 days after diagnosis; D 1y, T1D children 1 year after diagnosis; D 2y, T1D children 2 years after diagnosis; T1D, type 1 diabetes; ns, not significant. *p < 0.05, **p = 0.01, ***p < 0.01, ****p < 0.0001*

The expression of MCP-1 in islets has also been shown to increase concomitantly with the progression of insulitis in non-obese diabetic mice [166]. It has been speculated that macrophages positive for MCP-1 within the islets may attract immune cells and enhance the inflammatory "build-up". This may lead to further destruction of β-cells since intra-islet positive MCP-1 cells provide signals to extra-islet-located chemokines for leukocytes trafficking from vascular sites toward the islets [166, 167].

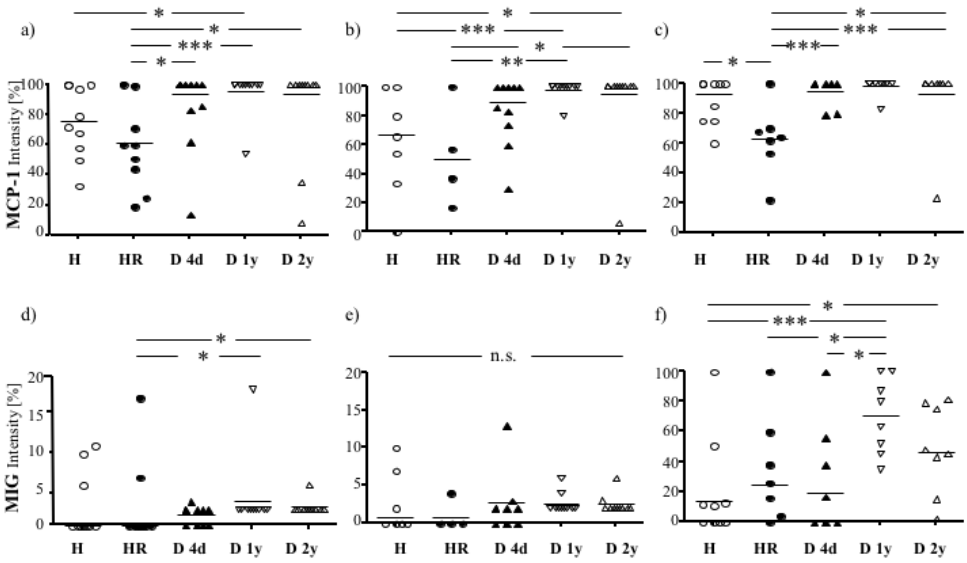

Figure 16 *The pro-inflammatory chemokines, monocyte chemoattractant protein (MCP)-1 and monokine upregulated by IFN-γ (MIG), secreted spontaneously (a, d) or from stimulation with either GAD65 (b, e) or PHA (c, f) presented as intensity (%). H, healthy; HR, high-risk; D 4d, T1D children 4 days after diagnosis; D 1y, T1D children 1 year after diagnosis; D 2y, T1D children 2 years after diagnosis; T1D, type 1 diabetes; ns, not significant. *p < 0.05, **p = 0.01, ***p < 0.01, ****p < 0.0001*

In paper II spontaneous secretion of pro-inflammatory TNF-α was lower in newly diagnosed T1D children, but increased again with disease duration (Fig. 16a). In contrast, and in concordance with the results seen in paper I, high-risk individuals had a low specific TNF-α secretion (Fig. 16b) and fewer responders from mitogen exposure compared to T1D and healthy children (not shown). TNF-α has been shown to play an important role in the pathogenesis of T1D development in NOD mice. In the NOD model, a wave of apoptosis in β-cells between days 17-20 have been postulated as responsible for the abrupt onset of insulitis at approximately three weeks of age [168, 169]. It has been shown that treatment with TNF-α before this 3-4 week checkpoint increased diabetes incidence in NOD mice, while treatment with anti-TNF-α during the same time period completely prevented diabetes during the one year study period [170]. However, treatment with TNF-α after the age of four weeks delayed onset, while administrating anti-TNF-α at the same age resulted in no decrease of diabetes onset, indicating that the time of TNF-α exposure is important for the outcome. To translate these ideas to a human model is more complex, as humans form an immensely heterogeneous population.

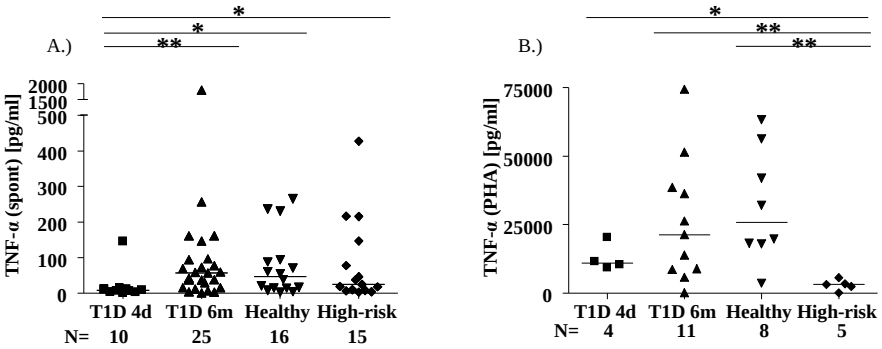

Figure 16 *Spontaneous (a) and phytohaemagglutinin (PHA) stimulated (b) secretions of Tumor Necrosis Factor (TNF)-α, measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test for two groups, and three or more groups using the Kruskal-Wallis test for unpaired observations. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01*

Studies of rheumatic models have shown IL-1β and IL-6 to be faster [171] and more dramatically increased, in comparison to TNF-α [172]. The pro-inflammatory cytokine IL-6 is foremost secreted in response to trauma such as burns or other tissue damage but has also shown, in a mouse model, to be essential for the resistance to *Streptococcus pnemoniae* [173]. It was therefore, perhaps, not so surprising that we did not find any differences in secretions, spontaneously or following autoantigen stimulation, between T1D children at onset or six months post diagnosis, healthy children and high-risk individuals (not shown). Logically, following these results, the quota of spontaneously secreted, as well as specific to GAD_{65} and HSP_{60}, TNF-α to IL-6, was suppressed in newly diagnosed T1D children (Fig. 17a-c). Interleukin-6 has been suggested to play a role in the apoptosis induction of the β-cells, in connection to both T1D and T2D. Even though IL-6 can contribute to apoptosis, it is not required nor enough to cause disease development (as reviewed by Kristiansen *et al* [174]). Considering the connection to trauma, differences in IL-6 secretions in response to damage of the pancreatic islets might be more obvious in the narrow window surrounding onset of the autoimmune attack, and then at disease onset return to baseline levels.

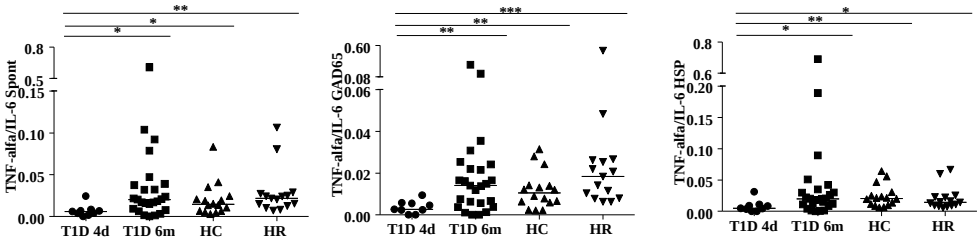

Figure 17 *The quota of Tumor Necrosis Factor (TNF)-α to interleukin (IL)-6 secretions spontaneously (a) as well as following Glutamic Acid Decarboxylase (GAD)65-protein (b) and the heat shock protein (HSP)60-peptide (a.a. 437-460) stimulation (c). Secretions measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test. The bars in each figure correspond to the median values. *=p<0.05, **=p<0.01*

Secretion of the inflammatory cytokine IL-1β exhibited a distinct pattern between the groups, both spontaneously as well as following mitogen stimulation. Newly diagnosed T1D children had a decreased spontaneous secretion when compared to secretions seen in high-risk individuals and healthy controls (Fig. 18a). This decrease, however, appears to be partially restored with disease duration. Following mitogen stimulation, the specific secretion was low both at high-risk and clinical T1D onset (Fig. 18b).

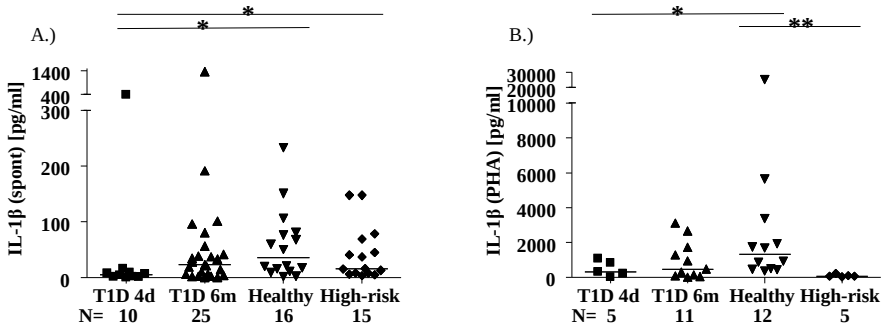

Figure 18 *Spontaneous (a) and phytohaemagglutinin (PHA) stimulated (b) secretions of interleukin (IL)-1β, measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test for two groups, and three or more groups using the Kruskal-Wallis test for unpaired observations. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01*

In contrast to our results, Meyers *et al* showed elevated serum levels of IL-1β in new onset T1D [175]. These results do not necessarily contradict each other, as serum levels are provided in a short period of time before sampling, while the *in vitro* results report the current condition and ability of action for the cells in circulation.

T1D duration

In contrast to most of the cytokines investigated in this thesis, spontaneously secreted and mitogen specific, RANTES were lower six months after the onset of T1D than at the diagnosis, and was also lower in healthy children and high-risk individuals (Fig. 19a-b). RANTES have been suggested to take part in the T1D pathogenesis, as single nucleotide polymorphisms (SNPs) related to decreased serum levels are associated with protection from T1D [176]. The decrease of RANTES from diagnosis to six months post diagnosis might mirror the decrease in secretion of inflammatory cytokines we see from the pre-diabetic phase to onset of T1D, considering that RANTES have a distinctly differing and delayed transcription and activation ([177] as reviewed by Song *et al* [178]).

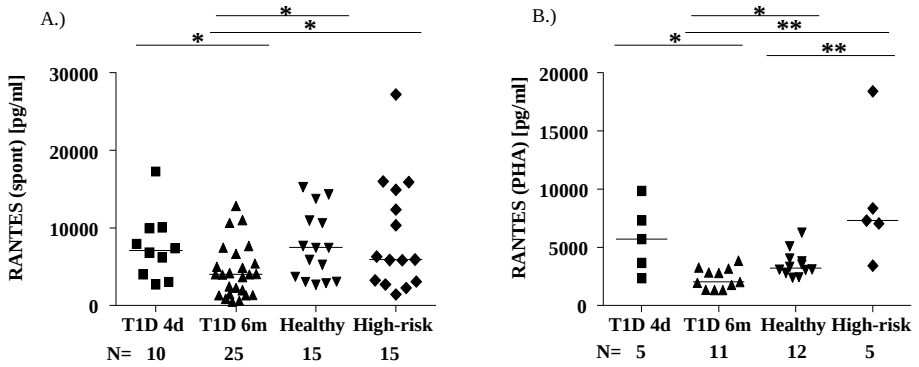

Figure 19 *Spontaneous and phytohaemagglutinin (PHA) stimulated secretions of Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) (a-b), measured by multiplex fluorochrome technology (Luminex) and compared by Mann-Whitney U-test for two groups, and three or more groups using the Kruskal-Wallis test for unpaired observations. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01*

Soluble and full length CTLA-4, FOXP3, TGFβand IL-10

Alterations of soluble and full length CTLA-4 during the pre-diabetic phase and T1D

The soluble splice form of CTLA-4 has been widely discussed in the past few years, but studies have failed to reach unanimity as to its role in the immune system. Even though it technically possesses all features but the membrane spanning section that would attach the molecule to the cell membrane, and thereby reasonably could function in a similar manner as the full length CTLA-4, an opposite role through competition has been discussed (see page 29-30). While some studies have indicated the presence of elevated levels of soluble CTLA-4 in autoimmune diseases such as Grave's disease, myasthenia gravis and systemic lupus erythematosus (SLE) [101, 107, 108, 110], we, however, observed a lower secretion of soluble CTLA-4 in T1D children compared to healthy and at-risk subjects, both in sera and spontaneous *in vitro* cultures as well as following stimulation with GAD_{65} or the mitogen PHA (Fig. 20a-d).

As mentioned previously, the +49A/G polymorphisms have been connected to reduction of soluble CTLA-4 in a gene dosage effect in T1D patients carrying the G-allele. Moreover, our group has previously shown that the CTLA-4 +49G/G genotype was associated with lower percentages of intracellular CTLA-4 positive $CD4^+$ cells and tended to have lower percentages of intracellular CTLA-4 positive $CD25^{high}$ cells compared to AA-genotype individuals [27]. Also, previous studies have shown associations between the genotypes +49G/G and CT60G/G, transferring the risk for autoimmunity and lower levels of soluble CTLA-4 transcripts [179, 180], while others failed to find associations between these genotypes and protein levels of soluble CTLA-4 [140, 181]. Thus, it does not seem unreasonable to speculate that a low ability to produce a potentially immune suppressive protein could be part of the disease pathogenesis. Different frequencies of genotypes between populations might explain variations in findings.

Secretions of soluble CTLA-4 in spontaneous cultures correlated with secretions detected in sera (Fig. 20e), indicating that both methods might be used.

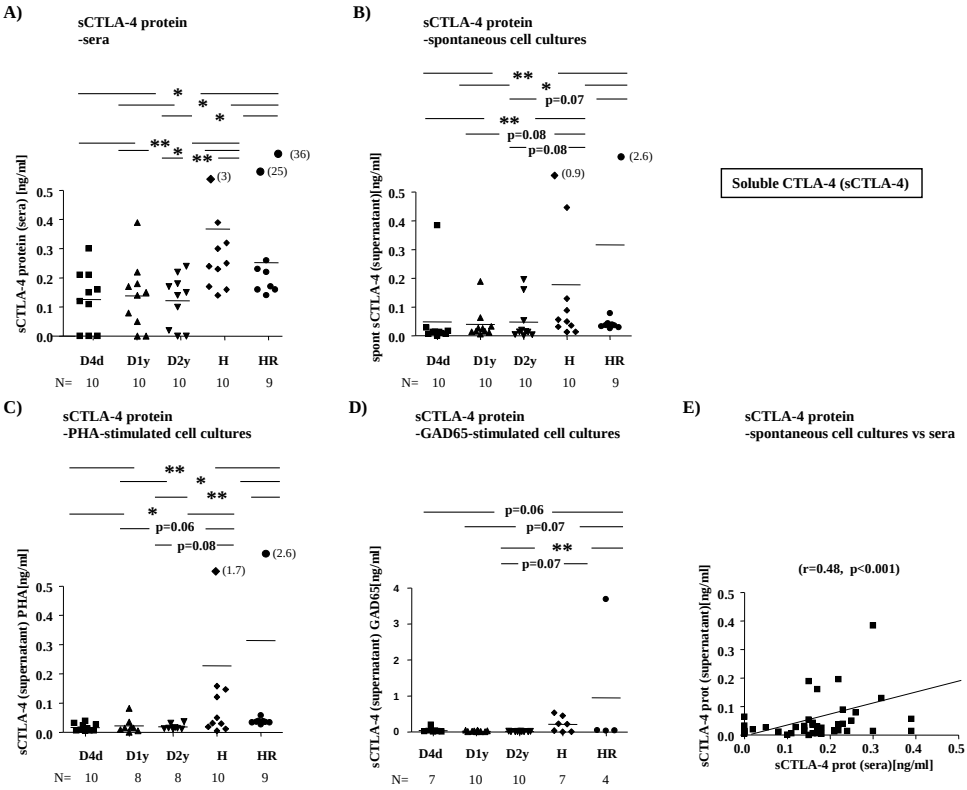

Figure 20 *Secretion of soluble CTLA-4 (presented as [ng/ml]) in sera (a), spontaneous cell culture supernatants (b), and in cell culture supernatants after PHA stimulation (c) or GAD65 stimulation (d) in type 1 diabetic, high-risk and healthy children. Soluble CTLA-4 protein secretion in sera correlated to secretion in spontaneous cell culture supernatants (r=0.48, p<0.001) (e). H = healthy, HR = high risk, D4d = T1D children four days after diagnosis, D1y = T1D children one year after diagnosis, D2y = T1D children two years after diagnosis. *=p<0.05, **=p<0.01*

To further support an alteration of soluble CTLA-4, secretion or processing, in disease course, T1D children displayed a positive correlation between expression of soluble CTLA-4 mRNA and actual protein secretion in cell culture supernatant at diagnosis that still tended to remain one year after diagnosis. This was not true for healthy children who, in contrast, showed a negative correlation (Fig. 21a-c).

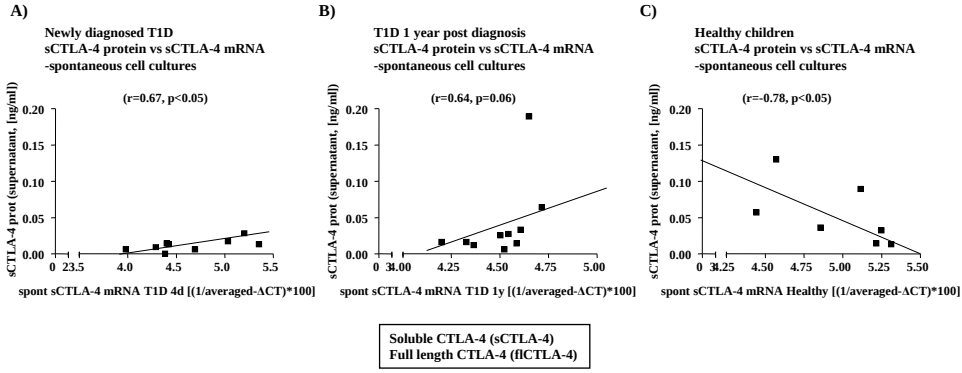

Figure 21 *Correlation between spontaneously secreted soluble CTLA-4 [ng/ml] measured in cell culture supernatants and spontaneously expressed soluble CTLA-4 mRNA [(1/averaged Δ-CT)*100] in T1D children four days (r=0.67, p<0.05) (a) and one year (r=0.64, p<0.05) (b) after diagnosis. In contrast, spontaneously secreted soluble CTLA-4 inversely correlated to soluble CTLA-4 mRNA in healthy children (r=-0.78, p<0.05) (c). T1D 4d = T1D children four days after diagnosis, T1D 1y = T1D children one year after diagnosis.*

In addition, the relationship between soluble CTLA-4 and full length CTLA-4 mRNA expression is consistent between the study groups even though the protein secretion is diminished in T1D children (Fig. 22b-f). Together these results could be regarded as a sign of a failure to transcribe the mRNA to protein or to keep the turnover of soluble CTLA-4 balanced to maintain suppressive circulating levels of the protein, rendering these children susceptible since they might not be able to control or suppress autoreactive T-cells in a sufficient way. For high-risk subjects no such correlation could be observed, possibly due to factors rendering part of the group to stay healthy while others develop disease. Furthermore, we also found that high-risk individuals had a significantly lower expression of soluble CTLA-4 mRNA following mitogen stimulation, as compared to T1D children from onset to one and two years after diagnosis as well as compared to healthy children. This might, as suggested above, be a sign of an exhausted immunological repertoire due to a prolonged general activation.

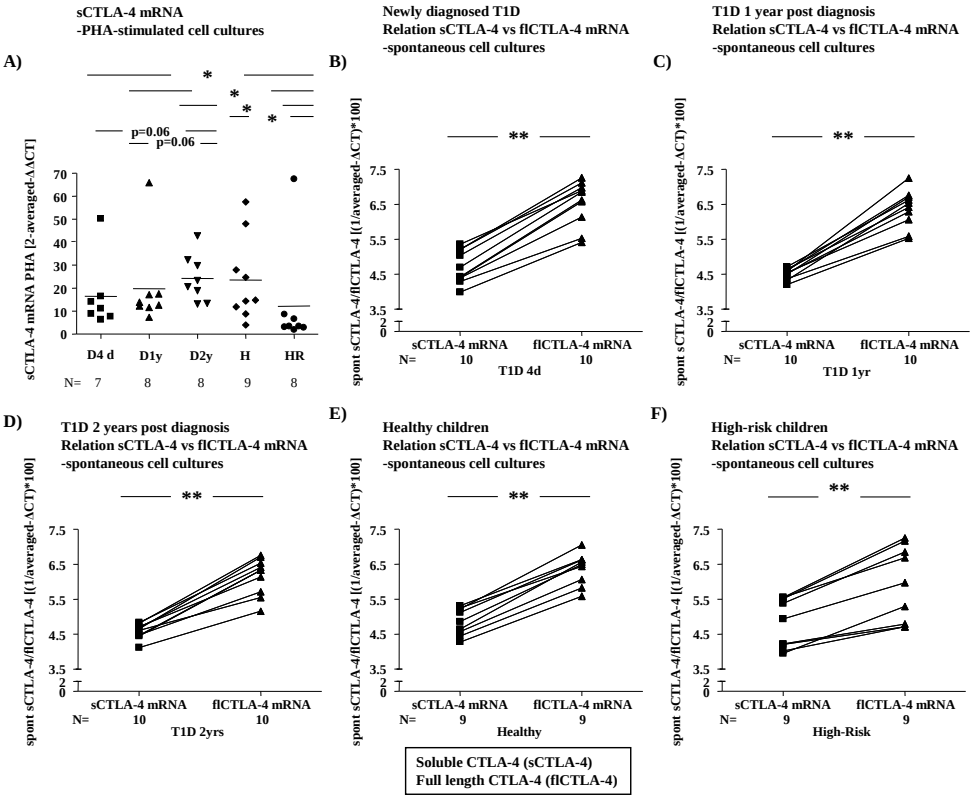

Figure 22 *(a) Specific PHA-induced soluble CTLA-4 mRNA expression, adjusted for spontaneous expression in type 1 diabetic, high-risk and healthy children, presented as [2 averaged ΔΔ-CT]. Spontaneous expression of soluble CTLA-4 and full length CTLA-4 mRNA, presented as [(1/averaged Δ-CT)*100] correlated in each sample, sharing the same pattern of higher full length CTLA-4 than soluble CTLA-4 mRNA, for all study groups; T1D four days after diagnosis (b), one year (p<0.01, (c) and two years (d) post diagnosis, healthy children (e) and high-risk individuals (f)). H = healthy, HR = high risk, D4d = T1D children four days after diagnosis, D1y = T1D children one year after diagnosis, D2y = T1D children two years after diagnosis. T1D 4d = T1D children four days after diagnosis, T1D 1y = T1D children one year after diagnosis, T1D 2yrs = T1D children two years after diagnosis.*
**=p<0.05, **=p<0.01*

Elevated mRNA expression of FOXP3, CTLA-4 and TGF-β at clinical T1D onset

We found that FOXP3, full length CTLA-4 and TGF-β (Fig. 23) were markedly up-regulated in their expression, both spontaneously and following GAD_{65} and mitogen exposure, in T1D children at time of diagnosis in comparison with still healthy high-risk individuals.

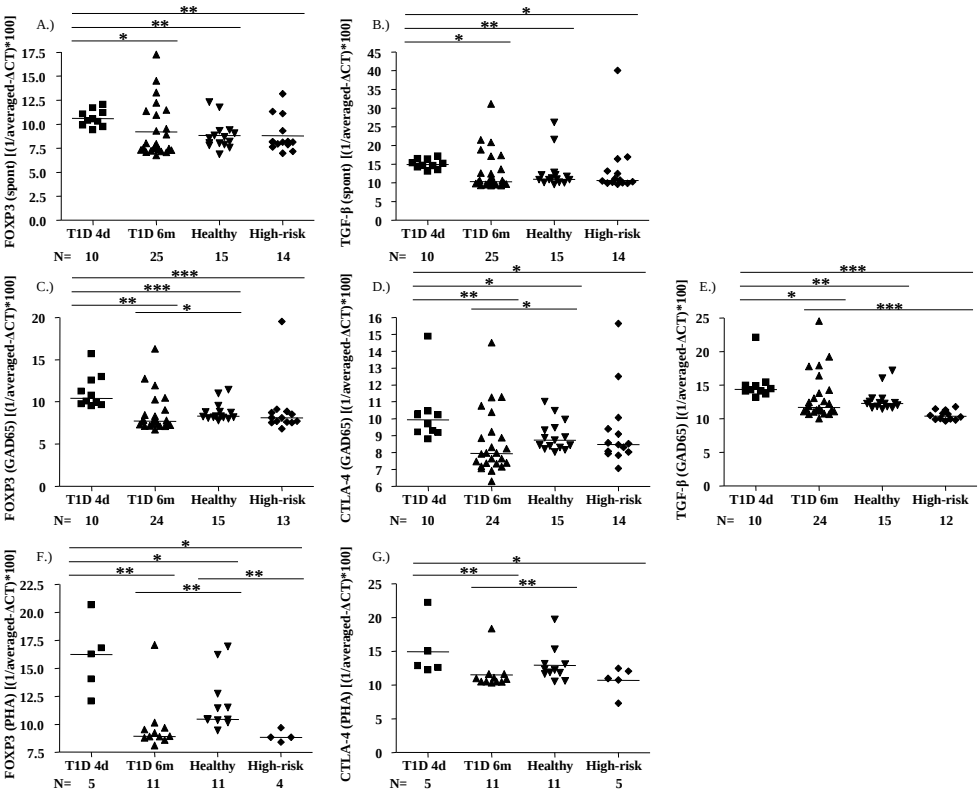

Figure 23 *Spontaneous forkhead box protein P3 (FOXP3) (a) and transforming growth factor (TGF)-β (b) mRNA expression. FOXP3 (c), cytotoxic T-lymphocyte associated protein 4 (CTLA-4) (d), and TGF-β mRNA (e) expression following stimulation with Glutamic Acid Decarboxylase (GAD)$_{65}$-protein. Phytohaemagglutinin (PHA) stimulated FOXP3 (f) and CTLA-4 (g) mRNA expression. FOXP3, CTLA-4, and TGF-β were detected by multiplex real-time RT-PCR and compared by Mann-Whitney U-test for two groups and Kruskal-Wallis test for unpaired observations, for three or more groups. The bars in each figure correspond to the median values. T1D 4d=newly diagnosed type 1 diabetic (T1D) children, T1D 6m=T1D children 6months post diagnosis. *=p<0.05, **=p<0.01, ***=p<0.001*

The up-regulation seen at T1D onset did, however, decrease markedly until at least six months post diagnosis and following mitogen stimulation FOXP3 and CTLA-4 mRNA were even lower than in healthy individuals. This is probably due to an exhaustion following the intense immune activation accompanying the disease onset and might reflect the loss of β-cells, hence the loss of target molecules for the autoimmune process. The strong up-regulation of Treg-associated markers at disease onset does not seem to be enough to restrict the autoimmune attack against the insulin secreting β-cells. Instead, this rather reflects a frustrated T-cell activation and

failure to suppress such activation in individuals proceeding from a pre-diabetic phase to T1D onset. These observations together suggest that individuals with an autoimmune attack against their beta-cells already long before disease onset, might have a differing responsiveness to immunological threats.

Low TGF-β mRNA expression during pre-diabetic phase and elevated protein secretion at clinical T1D onset

Expression of TGF-β mRNA was found to be lower in high-risk individuals, both spontaneously and following autoantigen stimulation (GAD_{65}), in comparison to T1D children from diagnosis to one and two years post diagnosis and also in comparison to healthy children. Also after mitogen stimulation, the high-risk individuals had a lower specific induction of mRNA, when compared to T1D children (Fig. 24a-c).

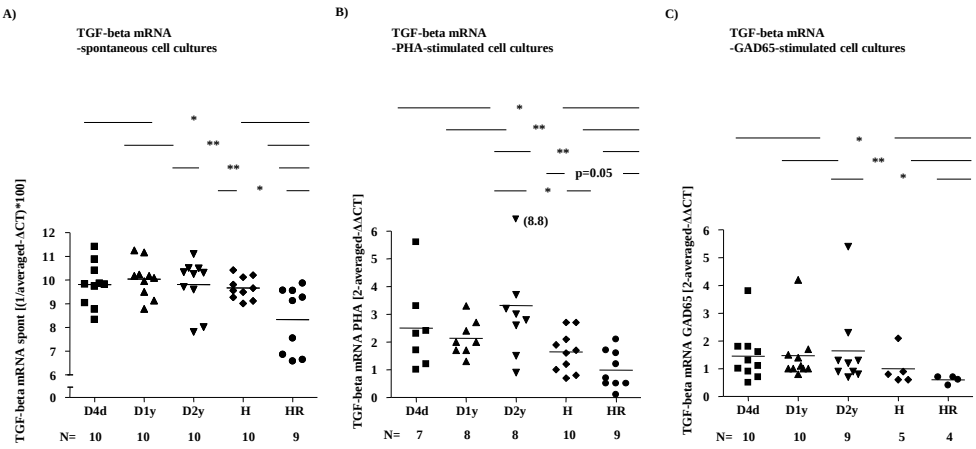

Figure 24 *Spontaneous [(1/averaged Δ-CT)*100] (a), PHA-induced [2 averaged ΔΔ-CT] (b) and GAD65-induced [2 averaged ΔΔ-CT] (c) TGF-β mRNA expression in type 1 diabetic, high risk and healthy children. *=p<0.05, **=p<0.01*
H = healthy, HR = high risk, D4d = T1D children four days after diagnosis, D1y = T1D children one year after diagnosis, D2y = T1D children two years after diagnosis.

These results are suggesting that a distortion in TGF-β expression occurs during the pre-diabetic phase. TGF-β have been considered to be important for natural (n)Treg commitment, but recent research suggested that it might instead antagonize the negative T-cell selection and in this way promote nTreg survival and thereby control

autoreactive T-cells [87]. A temporary disruption in TGF-β control within the general PBMC population of high-risk individuals, could thus possibly lead to fewer Tregs, which in turn could be a contributory factor to the development of T1D, rendering individuals unguarded towards potential threats such as Coxackie B viruses.

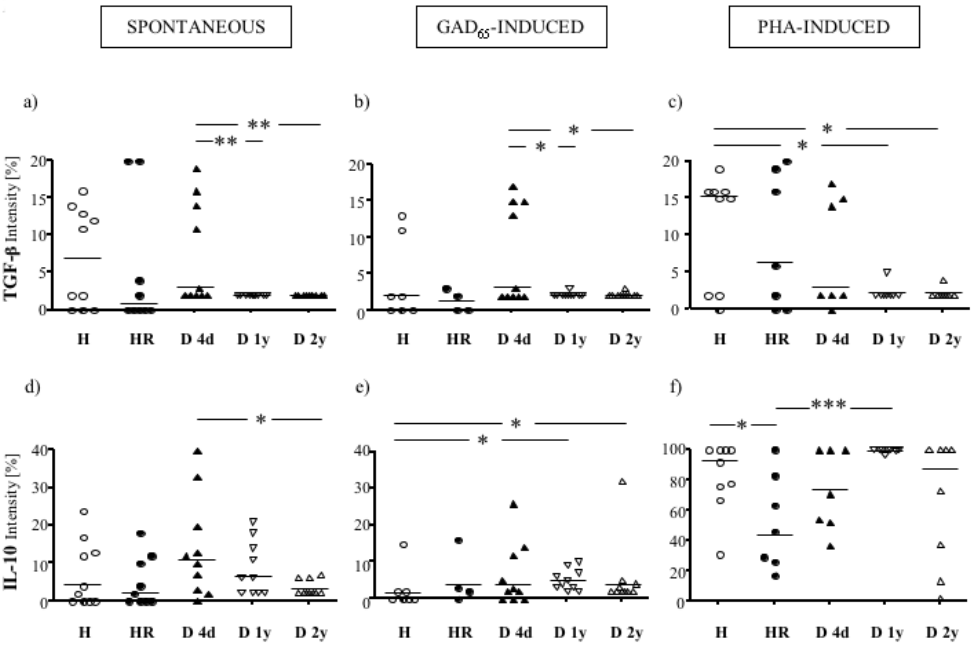

Figure 25 *The Th3-associated cytokines, TGF-β and IL-10, secreted spontaneously (a, d) or from stimulation with either GAD65 (b, e) or PHA (c, f) presented as intensity (%). H, healthy; HR, high-risk; D 4d, T1D children 4 days after diagnosis; D 1y, T1D children 1 year after diagnosis; D 2y, T1D children 2 years after diagnosis; Th, T-helper; TGF, transforming growth factor; GAD65, glutamic acid decarboxylase; PHA, phytohaemagglutinin; T1D, type 1 diabetes.*
**p < 0.05, **p = 0.01, ***p < 0.01*

Upon analysis of TGF-β protein secretions, our results correlated with those obtained from mRNA expression. Low spontaneous and GAD_{65} specific secretions in high-risk individuals were observed, as well as a further decrease in secretions seen from clinical T1D onset to one and two years after diagnosis (Fig. 25a-b). Mitogen induced secretion at one and two years post T1D diagnosis were significantly lower from that in healthy children (Fig. 25c). Also, spontaneous IL-10 (Fig. 25d) decreased from diagnosis to 2 years after the onset of T1D. In contrast, secretion of IL-10 by GAD_{65} stimulation was higher at 1 and 2 years duration compared with healthy children (Fig. 25e). High-risk children secreted less IL-10 from stimulation with PHA compared with T1D children 1 year after the onset, and with healthy children (Fig.

25f). Moreover, spontaneous IL-10 secretion correlated with C-peptide levels at clinical onset of T1D (Fig. 26), *i.e.* individuals with a better clinical status (retained C-peptide), had a higher IL-10 secretion. IL-10 contributes to the suppression of Th1-associated cytokines and induction of B cell activity. We have learned that TGF-β plays an essential role for the generation of FOXP3+ inducible Treg cells as well as an important effect on natural Treg cells [33]. TGF-β is found at high levels at inflammatory sites [34] and increased IL-10 secretion has been observed in longstanding (6 months to 5 years) T1D patients [27], supporting our data.

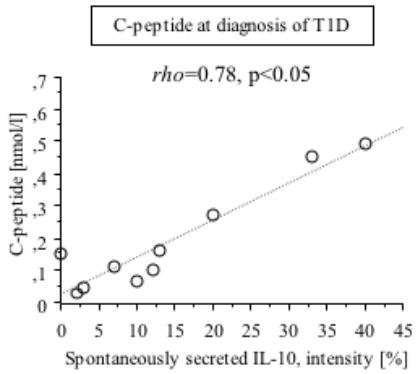

Figure 26. Spontaneously secreted interleukin (IL)-10 was related to secretion of C-peptide at onset of disease

T1D development in high-risk individuals

To further investigate the pre-diabetic phase, we divided the high-risk group into those that have progressed to clinical diagnosis and those that are still undiagnosed. We found that those high-risk individuals that later progressed to T1D, were those that expressed the lowest mRNA levels, following GAD_{65} exposure, of the Treg-associated markers FOXP3, CTLA-4 and TGF-β (Fig. 27a-c). This indicates a lower ability to activate, and thereby suppress, in response to one of the major putative autoantigens in T1D. Moreover, we found spontaneous secretion of the chemokine MIP-1β to be higher in the individuals that later developed T1D (Fig. 27d). Thus, it seems as though the high-risk individuals that developed T1D were more prone to a pro-inflammatory and chemotactic response, probably connected to recruitment and activation of leukocytes and release of other pro-inflammatory cytokines. This further indicates an altered behavior of the immune system already years before T1D diagnosis.

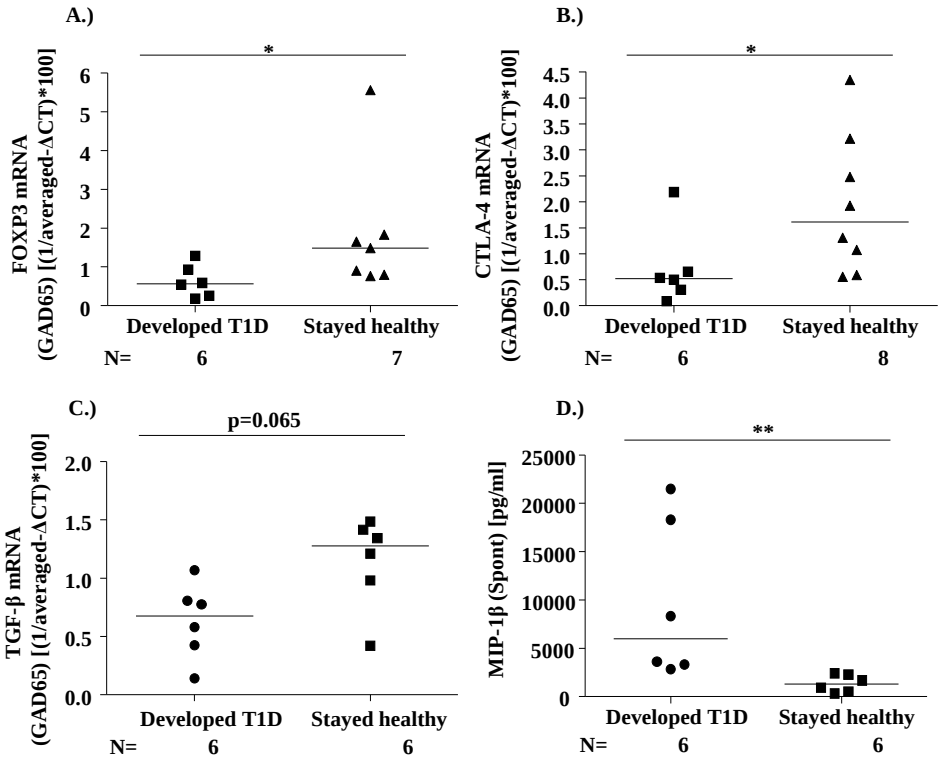

Figure 27 *Forkhead box protein P3 (FOXP3) (a), cytotoxic T-lymphocyte associated protein 4 (CTLA-4) (b), transforming growth factor (TGF)-β (c) mRNA expressions, following stimulation with Glutamic Acid Decarboxylase (GAD)65-protein and spontaneous MIP-1β secretion (d) in high-risk individuals that remained healthy in comparison to those that later developed type 1 diabetes (T1D). FOXP3 and CTLA-4 were detected by multiplex real-time RT-PCR, secretions measured by multiplex fluorochrome technology (Luminex), and compared by Mann-Whitney U-test for two groups and Kruskal-Wallis test for unpaired observations, for three or more groups. The bars in each figure correspond to the median values. *=p<0.05, **=p<0.01*

We also saw that the high-risk individuals who subsequently developed T1D were those that exhibited the lowest soluble CTLA-4 mRNA expression in response to mitogen stimulation; however, they also showed higher spontaneous expression of full length- and soluble CTLA-4 mRNA than individuals who remained healthy (Fig. 28a-c). This elevated general activation might reflect an early sign of an on-going immunological process, as part of the pre-diabetic phase, which in turn might explain the low ability to respond to mitogen with induction of soluble CTLA-4.

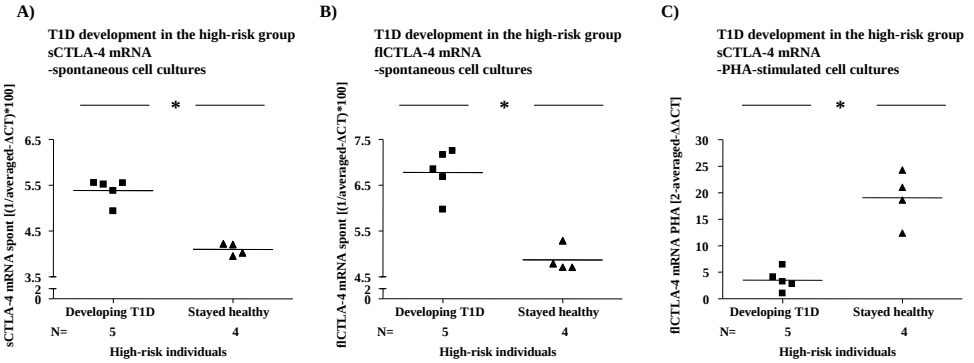

Figure 28 *Expression of spontaneous soluble CTLA-4 (a), full length CTLA-4 mRNA [(1/averaged Δ-CT)*100] (b) and PHA-induced full length CTLA-4 mRNA [2 averaged ΔΔ-CT] (c) in high-risk individuals that developed T1D or remained undiagnosed. *=p<0.05*

Supporting these speculations, we previously showed lower levels of IL-10 following mitogen stimulation in high-risk individuals [153] and also that T1D children responded with increased IFN-γ expression but rarely IL-4 expression to the autoantigen GAD_{65} when compared to healthy children [182]. Together these results suggest that these individuals who proceed to disease might have an impaired ability to suppress the immune response.

Mitogen-activation

One interesting feature seen for various markers was that high-risk individuals often had a poorer response to stimulation with the mitogen PHA, in comparison to healthy and T1D children. For example, mitogen stimulation strongly elevated the levels of IL-10 secreted from PBMC of healthy as well as T1D children, but to a lower extent in high-risk individuals. A low mitogen-induced response in high-risk individuals was also true for TNF-α, MCP-1, and IL-1β secretion. One could speculate that this might be due to the fact that there already seems to be a strong background activation of these cells and that this is a sign of exhaustion. However, this further raises the question whether one should consider using a different type of positive control for stimulation. While one could speculate that general mitogen activation will give us an indication of the condition of the PBMC population of the different patient groups, one could argue that a positive control like tetanus toxoid will activate cells in a more similar fashion to the autoantigens implicated in T1D pathogenesis.

Expansion of cryopreserved CD4+CD25+CD127lo/- cells

Establishment of T-cell tolerance takes place both centrally in the thymus and through various mechanisms in the periphery [183]. The strongly suppressive regulatory T-cells (Tregs) are bridging central and peripheral tolerance mechanisms through thymic derived and peripherally induced subsets. A common description of the Treg phenotype is the concurrent expression of CD4 and high constitutive expression of the alpha chain of the IL-2 receptor (CD25) [82]. To further identify a regulatory population devoid of activated effector T-cells, a number of markers have been described, most notably absent or low expression of the IL-7 receptor CD127 [73, 74]. However, pinpointing a pure Treg population is a complex task in humans due to the lack of a specific and unique Treg marker together with the heterogeneity of this population. In the research field of T1D, where study subjects are often children, blood volumes available for T-cell studies are limited and samples given must be carefully handled and used to its full extent. Cryopreservation of peripheral blood mononuclear cells (PBMC) in liquid nitrogen allows for batch analysis of samples, but the number of Tregs that can be recovered is limited (~5-7% of CD4+ T-cells) [184]. *In vitro* expansion of these cells would therefore be most valuable for biological investigations, and, possibly, for adoptive cell therapies.

An important concern regarding flow cytometry analysis of cryopreserved cells is that the expression of surface- and intracellular markers could be affected by the cryopreservation and thereby alters the phenotypes of the studied cells. Hence, we sought to establish the cryostability of Tregs, *i.e.* of markers associated with this group of cells. To do this we analysed the Treg marker FOXP3 in the $CD4^{+}CD25^{hi}$ population. We were pleased to find that the expression of FOXP3 was not altered regarding percentage of expressing cells nor MFI (Fig. 29a-b). Neither did MFI of CD25 change markedly from sampling to post-cryopreservation (Fig. 29c). Others have reported a somewhat diminished suppressive function directly upon thawing, but this was restored upon expansion. Further, if Tregs were expanded prior to cryopreservation, the suppressive effect was unaffected upon thawing [185]. These results suggest that the highly interesting Tregs are stable for applications such as flow cytometry and cell sorting, following cryopreservation and thawing. Importantly, others have shown that isolated Tregs can survive cryopreservation [185].

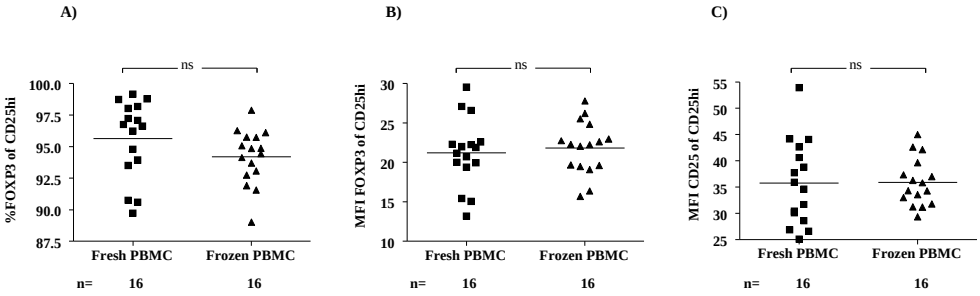

Figure 29 *Percentage of FOXP3 expressing cells (a) mean fluorescence intensity (MFI) of FOXP3 expression (b) and MFI of CD25 expression (c), in $CD4^{+}CD25^{hi}$ cells before and after cryopreservation of isolated peripheral blood mononuclear cells.*

While the so called classic Treg gating strategy, based on the concurrent expression of CD4 and the highest expression of CD25, comprise only about 1-2% of the $CD4^{+}$ cells, Liu *et al* [73] demonstrated that Tregs defined by the concurrent expression $CD4^{+}CD25^{+}CD127^{lo/-}$, as gated in figure 9, comprised a larger cell number but were just as suppressive. Furthermore it has been shown that the exclusion of $CD127^{hi}$ expressing cells, as done with this type of gating, allowed for isolation of Tregs without contamination of memory effector cells [141]. Beside the above mentioned findings, we found this gating strategy for Tregs (Figure 1) to be solid and it was therefore chosen over the so called classic gating strategy.

We were able to achieve powerful expansion of Tregs from all individuals, independent of study group, even when starting with as few as four thousand sorted Tregs (Fig. 30a). These results highlight the effectiveness of the method. A significant increase in Treg numbers, despite a highly limited starting material, is an important outcome, should autologous Treg therapy ever be the goal.

Although there were no statistical differences in fold increase of Tregs between the study groups (Fig. 30c), Tregs of healthy study subjects might be more prone to a higher fold increase than Tregs of T1D subjects based on the display of higher fold expansion in half the group in comparison to expansions seen from T1D and high-risk individuals. A previous study, asserted that fold expansion of $CD4^{+}CD127^{lo/-}$ $CD25^{+}$ T-cells were negatively correlated to age [141]. However, we could not see any such correlation, nor a positive, between ages and fold expansion in our study cohort. Certainly it was not the youngest subjects in the healthy group that expanded

the most. Further, no difference in fold expansion of $CD4^+CD25^-$ T-cells between the groups was observed. This indicates that despite the higher proportion of $CD4^+CD25^-$ observed in the T1D cohort, there does not seem to be an altered proliferation rate to engagement of CD3 and CD28.

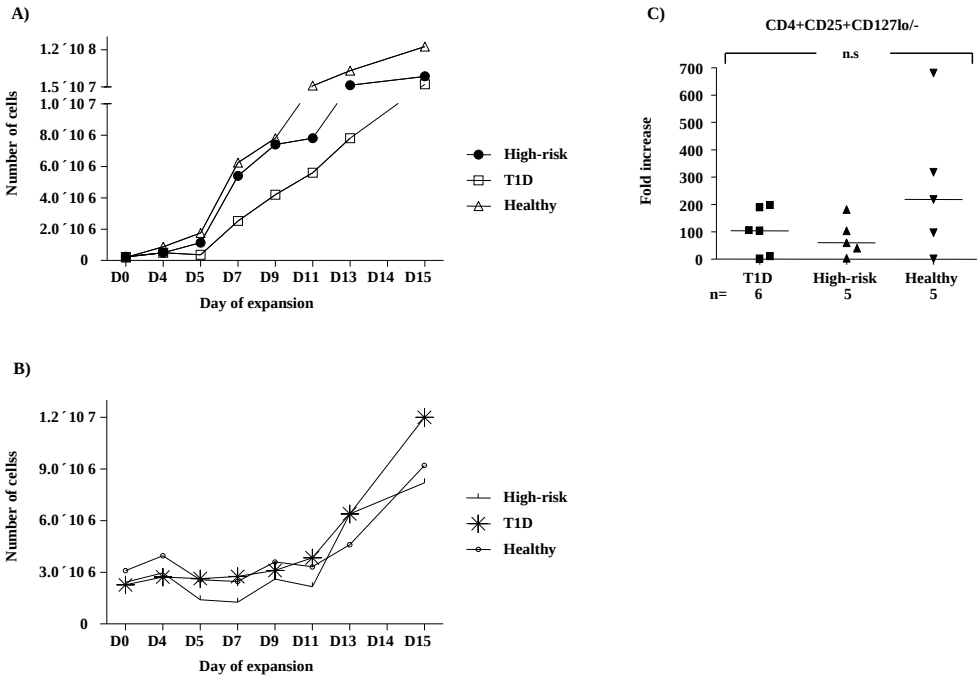

Figure 30 *Representative expansion curves of sorted a) $CD4^+CD25^+CD127^{lo/-}$ Treg and b) $CD4^+CD25^-$ cells. Fold increase c) of Treg did not differ significantly between T1D, healthy and high-risk individuals, even if observations for healthy individuals visually appeared to be higher.*

Further, we observed a skewing of the $CD4^+$ T-cell composition towards a larger proportion of $CD4^+CD25^-$ cells in T1D children (Fig. 31) which could be explained as part of their failure to protect against different threats. This could be speculated since they hold a larger proportion of cells that upon engagement of CD3 and CD28 induces fewer FOXP3 expressing cells with lower FOXP3 intensity, in comparison to $CD4^+CD25^+CD127^{lo/-}$ cells.

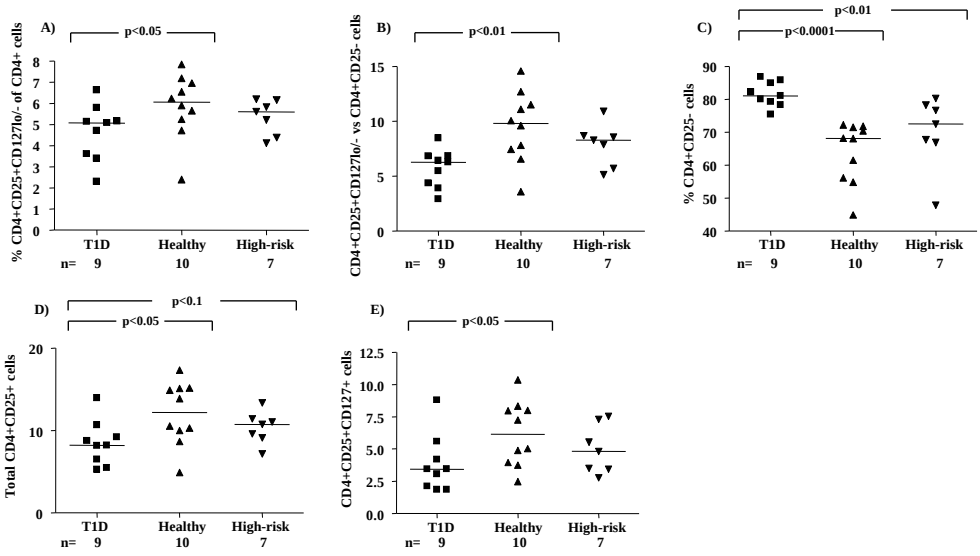

Figure 31 *The percentage of CD4⁺CD25⁺CD127lo/- Treg in the CD4⁺ fraction (a) CD4⁺CD25⁺CD127lo/- quota comparing CD4⁺CD25⁺CD127lo/- to CD4⁺CD25⁻ (b), percentages of CD4⁺CD25⁻ (c), percentage of the total CD4⁺CD25⁺ cell count (d) and fractions of CD25⁺CD127⁺ (e) in the CD4 positive population.*

Following expansion, almost all of the sorted CD4⁺CD25⁺CD127lo/- Tregs expressed FOXP3 as compared to expanded CD4⁺CD25⁻ responder cells, where a big but significantly lower percentage expressed FOXP3 (Fig. 32a-c). Not only did a higher percentage of sorted and expanded Tregs express FOXP3 compared to CD4⁺CD25⁻ T-cells post expansion (Fig. 32d), but they also exhibited significantly higher intensity of FOXP3 (Fig. 32e-f). This encourages the use of sorted CD4⁺CD25⁺CD127lo/-Tregs for expansion, as they appear to generate cells with strong CD25⁺FOXP3⁺ expression.

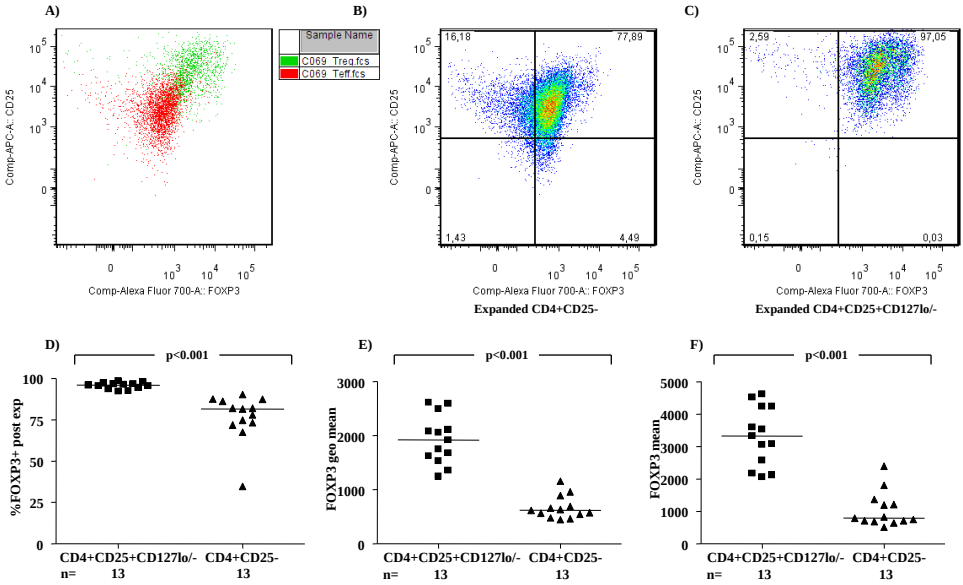

Figure 32 *A big part of the sorted CD4⁺CD25⁻ cells had become FOXP3⁺CD25⁺ after 15 days of expansion, but the cells expanded starting with sorted CD4⁺CD25⁺CD127lo/- Tregs had a higher FOXP3 expression intensity, as well as a higher percentage of positive cells. Figure (a) shows a representative overlay of FOXP3 expression in expanded cells starting with sorted CD4⁺CD25⁺CD127lo/- and CD4⁺CD25⁻ of the same individual, and in (b) FOXP3⁺CD25⁺ expression after expansion of sorted CD4⁺CD25⁻ and (c) after expansion of sorted CD4+CD25⁺CD127lo/-. Cumulative data of the expansion of sorted CD4⁺CD25⁺CD127lo/- T cells d). Mean fluorescent intensity (MFI) of FOXP3, both expressed as (e) geometrical mean and (f) mean from expanded CD4⁺CD25⁺CD127lo/- cells, compared to expanded cells starting with sorted CD4⁺CD25⁻.*

Statistically, we did not see any difference between the study groups, in the percentage of FOXP3 expressing cells in the expanded Treg cultures. However, half of the observations for T1D individuals were higher than all the other observations and T1D also showed a tendency for higher FOXP3 intensity (Fig. 33a). No such differences were seen for the sorted and expanded CD4⁺CD25⁻ cells. Taken together, although T1D may be associated with a smaller Treg proportion, they were able to achieve a great Treg expansion and even acquired higher FOXP3 expression than healthy individuals (33b-c). Considering the variation of the T-cell composition between the groups, one might hypothesize that the Tregs of T1D are predominantly naïve or resting Tregs which could explain their good expansion potential and higher FOXP3 upregulation [75]. With this in mind, one could speculate that T1D subjects possibly could benefit from *in vitro* expansion and re-introduction of the autologous Tregs to keep the immunological process towards the pancreatic β-cells in check. Due

to limited sample sizes, we were unable to show functional data of the expanded $CD4^+CD25^+CD127^{lo/-}$ Tregs to establish the efficaciousness of the method and the possibility to use the expended cells as Tregs in future applications.

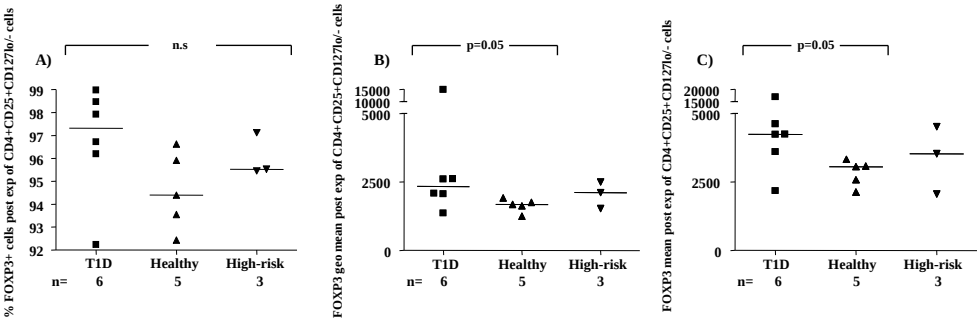

Figure 33 *The percentage of FOXP3 expressing cells in the expanded $CD4^+CD25^+CD127^{lo/-}$ Treg cultures (a) FOXP3 MFI, both expressed as (b) geometrical mean and (c) mean, post expansion of $CD4^+CD25^+CD127^{lo/-}$.*

SUMMARY AND CONCLUSION

The complexity and the multifactorial background of T1D craft a highly interesting, but elusive, research area. Working with humans one deals with a vastly heterogeneous study population, further adding to the complexity of the research area, in comparison to the homogenous inbred mouse models, such as the NOD mouse. However, not only the strong heterogeneity of humans set up obstacles in the T1D research area, but also the fact that the possibilities for *in vivo* and *in situ* investigations are limited. A common way of studying the immune system of humans is through the isolation of PBMC from blood samples. With isolated cultures we can minimise the impacting variables and control the environment, when investigating the functions and characteristics of the peripheral immune cells. Study models deprived of interference from the surrounding environment, however also means, that we are looking at a small section of the picture. We always need to bear in mind that what we are looking at are the effects of cells outside the site of action, and that the profile present in the pancreatic islets, directly contributing to the β-cell destruction might look different.

In our quest to try and characterise the immunological profile from the pre-diabetic phase to onset of, and further on during the course of T1D, we found that high-risk individuals showed to differ in expressions and secretions, of a variety of immune markers, from that seen in healthy and T1D children. Furthermore; high-risk individuals that later developed T1D had a lower mRNA expression of the regulatory associated markers FOXP3, CTLA-4 and TGF-β, following stimulation with the major autoantigen GAD_{65}, in combination with higher secretions of the chemotactic pro-inflammatory cytokine MIP-1β. Taken together, these results indicate that the high-risk individuals that developed T1D had a lower ability to activate and thereby suppress, in response to one of the major putative autoantigens in T1D, and also seemed to be more prone to a pro-inflammatory and chemotactic response, probably connected to recruitment and activation of leukocytes and release of other pro-inflammatory cytokines. These alterations seen in high-risk individuals may play a pivotal role for the development of T1D.

We found low levels of circulating soluble CTLA-4 in sera together with a positive correlation between soluble CTLA-4 protein secretion and mRNA expression in T1D,

in parallel to a negative relation in healthy individuals. Moreover, low C-peptide was accompanied by low mitogen-induced soluble CTLA-4 protein, and vice versa, pointing to a link between clinical process, *i.e.* β-cell degradation and ability to secrete the regulatory molecule soluble CTLA-4 upon mitosis. As recombinant soluble CTLA-4 Ig has been shown, both *in vitro* and *in vivo*, to inhibit T cell proliferation and is used to treat autoimmune disorders such as rheumatoid arthritis [35], it is interesting to speculate whether treatment with soluble CTLA-4 Ig could be beneficial in T1D. However, to be able to delay or prevent β-cell destruction, such intervention would have to take place during the pre-diabetic phase. While the suppressive potential of soluble CTLA-4 seems to be accepted, its role in disease model remains elusive.

In addition to a markedly altered immune profile already during the pre-diabetic phase, T1D seems to present with an intense up-regulation of regulatory and pro-inflammatory markers and a suppression of Th1 and Th2-associated immunity. This up-regulation of regulatory markers, however, seems to occur too late in the immunological process to suppress the autoimmune attack directed against the pancreatic β-cells, and is probably reflecting the strong activation seen at onset of disease, rather than a cause of disease.

During the course of T1D, less and less β-cells can be expected to be left in the pancreatic islets, until finally a total lack of β-cells is the end-point. Following this reasoning, less and less stimulus to drive the autoimmune process should be present in the course of disease duration. This might be the explanation to why we see a decrease of the, during the pre-diabetic phase, elevated Th1-associated and pro-inflammatory cytokines and chemokines, along with onset and duration of disease. It could also reflect why we see a decrease in IL-10 and TGF-β, from onset of disease towards two years of duration.

In paper IV we sought to investigate the cryostability of Treg associated markers to efficiently expand Tregs and to detect any difference in T cell number and composition among the studied subjects. We found cryopreserved PBMC to be feasible for flow cytometric analysis, sorting and expansion of Tregs, as marker expression were stable through cryopreservation and thawing and an efficient fold

expansion with preserved high FOXP3 expression was achieved. Further, our study suggests that T1D is associated with a lower percentage of Tregs, however, the ones they have expand well and even acquire a higher FOXP3 expression. Whereas we found an altered composition of $CD4^+$ subsets, biased towards a higher $CD4^+CD25^-$ ratio to $CD4^+CD25^+CD127^{lo/-}$ Tregs, the importance of said alteration remains to be shown. Considering these results, it seems likely, as mentioned above, that the strong upregulation of FOXP3, TGF-β and CTLA-4 mRNA at T1D onset, is a sign of activation rather than regulation.

Future perspectives

As we see alterations in the immunological profile of high-risk individuals, especially between high-risk individuals that later progressed to clinical T1D and those still undiagnosed, years before diagnosis; it would be highly interesting to be able to characterise and monitor a larger cohort of high-risk individuals during a prolonged period of time. If this could be done, one might be able to describe differing features between those individuals that remain undiagnosed and those that progress to clinical T1D.

Soluble CTLA-4 is an interesting protein, displaying suppressive immunological functions in *in vitro* systems and as a recombinant Ig molecule in disease treatment. However, the varying findings of elevated or decreased levels in different autoimmune models are elusive. As we see low levels of the protein at T1D onset that also remain low at least up to two years following diagnosis, the decrease does not seem to be just connected to the inflammatory process seen at T1D onset. It would be of interest to study the dose-response relationship of soluble CTLA-4 on effector cells, and also to compare this between children diagnosed with T1D, high-risk individuals and healthy children. Further it would be interesting to detect soluble CTLA-4 during the pre-diabetic phase and up to diagnosis; all to try and find the checkpoint where the levels start to decrease. If this could be done, it might be possible to answer to why the levels go down.

The results gained from flow cytometric analysis, sorting and expansion of Tregs characterised as $CD4^+CD25^+CD127^{lo/-}$ cells, indicated a lower quota of this population

in comparison to $CD4^+CD25^-$ cells in T1D patients. However, sorted Tregs from T1D children expanded well. Unfortunately, due to limited sample size, we were not able to perform any functional studies on the expanded cells. In a future setup, it would be interesting to be able to investigate the suppressive functions of Tregs from T1D and high-risk individuals, prior to, as well as following, expansion, to gain further knowledge about the condition and potential of this important cell type, and its potential role in disease pathogenesis.

T1D is a serious disease that requires lifelong treatment. Therefore, clinical trials set to preserve β-cell function and delay or prevent clinical onset of T1D, are very important. Hence, immunological studies of the pre-diabetic and the diabetic phase, are essential to pinpoint targets and characterise regulatory cells which might be used in therapeutic approaches.

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A man content to go to heaven alone will never go to heaven

Boethius

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