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The Importance of CTLA-4 and HLA Class II for Type 1 Diabetes Immunology

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During the course of the research underlying this thesis, Carl-Oscar Jonson was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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To mom and dad,
And everybody else who have believed in me

"I think now, looking back, we did not fight the enemy;

we fought ourselves. The enemy was in us."

Private Chris Taylor, "Platoon"

Abstract

Type 1 Diabetes (T1D) is a serious chronic disease that results from an autoimmune destruction of the insulin-producing beta cells. Sweden has the second highest incidence of T1D in the world, and it affects more and more children each year. Genes controlling key functions of the immune system regulation of autoimmunity has been associated to T1D. Polymorphism in the Human Leukocyte Antigen (HLA) Class II is a major risk determinant for T1D but also Cytotoxic T lymphocyte Antigen 4 (CTLA-4) polymorphism can affect predisposition. Immune responses towards Glutamic Acid Decarboxylase 65 (GAD65), Insulin, insulinoma-associated antigen 2 (IA-2) and Heat Shock protein 60 have all been implicated in T1D pathogenesis.

We aimed to study the effect and role of CTLA-4 and HLA Class II in the T1D immunity. By focusing on the immune responses associated to T1D in healthy children with risk genotypes we aimed to study immunological effects of T1D risk.

We found that HSP60-peptide induced a higher IFN-γ response in subjects with risk associated CTLA-4 +49GG allele while GAD_{65} induced IL-4 secretion was lower in risk subjects. Individuals with T1D neutral HLA showed higher IFN-γ responses to GAD_{65} than DR3-DQ2 and DR4-DQ8 positive children. We did also detect that T1D patients have reduced IFN-γ responses to GAD_{65} compared to healthy children. Interestingly, HLA and CTLA-4 risk genotype seem to reduce those responses to become similar to responses of T1D patients. We also found that CTLA-4 and HLA risk is associated to reduced percentages of lymphocytes expressing intracellular CTLA-4 in healthy children. In another study we recorded maintained levels of CTLA-4 and TGF-β mRNA responsiveness to GAD_{65} in recent onset T1D patients receiving ECP treatment although clinical outcome was certainly limited.

In conclusion, HLA Class II risk genes but also CTLA-4 +49A/G to some extent, influence CTLA-4 capacity and T1D protective antigen-specific responses in a manner that might explain the genes' predisposing and pathogenic capability.

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Abbreviations

AIRE	Autoimmune regulator
AP	Alcalic Phosphatase
APC	Antigen Presenting Cell
APC	Allophycocyanin
CD	Celiac Disease
cDNA	Complementary DNA
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic Cell
DNA	Deoxyribonucleis acid
ECP	Extracorporeal photochemotherapy
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immuno-spot
FITC	Fluorescein isothiocyanate
GADA	GAD65 antibody
HLA	Human Leukocyte Antigen
HSP60	Heath shock protein 60
IDO	Indoeamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukin
ITPR3	Inositol 1,4,5-Triphosphate Receptor 3
MHC	Major Histocompability Complex
MOP	Methoxypsoralen
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein
PHA	Phytohaemaglutinin
PTPN22/LYP	Lymphoid protein tyrosine phosphatase
PVDF	Polyvinylidene Difluoride
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
SLE	Systemic Lupus Erythematosus
T1D	Type 1 Diabetes
TB	Tuberculosis
TCR	T cell receptor
Th	T helper (cell)
TNF	Tumor necrosis factor

Original publications

This thesis is based on the following papers, referred to in the text according to their roman numerals:

I. Carl-Oscar Jonson, Åke Lernmark, Johnny Ludvigsson, Elizabeth A Rutledge, Ari Hinkkanen, Maria Faresjö.

"The importance of CTLA-4 polymorphism and Human leukocyte antigen genotype for the induction of diabetes-associated cytokine response in healthy school children" (*Pediatric Diabetes 2007* Jul*:*8(4):185)

II. Carl-Oscar Jonson, Maria Hedman, Maria Karlsson Faresjö, Rosaura Casas, Jorma Ilonen, Johnny Ludvigsson, Outi Vaarala for the ABIS study group

"The association of CTLA-4 and HLA Class II Autoimmune Risk Genotype with Regulatory T-cell Marker Expression in 5-year-old Children" (*Clinical and Experimental Immunology* 2006 Jul;145(1):48-55.)

III. Carl-Oscar Jonson, Mikael Pihl, Caroline Nyholm, Corrado M Cilio, Johnny Ludvigsson, Maria Faresjö.

"Regulatory T-cell associated activity in Photopheresis-induced Immune tolerance in Recent Onset Type 1 Diabetes Children" (Submitted to *Pediatric Research*)

IV. Carl-Oscar Jonson, Brian Van Yserloo, Johnny Ludvigsson, Åke Lernmark, Maria Faresjö.

"Children with CTLA-4 +49A/G and HLA class II risk alleles show similar Th1-like response towards GAD65 as children with manifest type 1 diabetes" (manuscript)

Review of the literature

Introduction

Type 1 Diabetes (T1D) is a complex and serious disease. It affects children from very young age and has implications for the whole family. For example, life-long treatment of insulin, constant glucose monitoring and risk of life threatening hypoglycaemia and complications. These are burdens associated with the disease. As to this moment, the only treatment available is symptomatic. However, there are several promising clinical trials in progress and hopefully some of these will reach the goal of preventing Type 1 Diabetes.

From a research perspective Type 1 Diabetes is very interesting. Although international conferences in T1D bring together several thousand researchers involved in the area that have devoted many years to this field, many questions remain unanswered. What causes T1D? There are several ways of inducing T1D in animal models, but we still don't know what causes the disease in man. There are also several ways of curing T1D in animal models, but unfortunately none of these has yet been translated to human therapies. What is quite certain is that T1D is a multifactorial disease. This is what makes the research both hard and challenging.

In my work I have been studying some fundamental mechanisms of immune function in relation to T1D. Common for several autoimmune diseases, Human Leukocyte Antigen (HLA) and Cytotoxic Lymphocyte Antigen 4 (CTLA-4) are critical for a healthy immune system. Interference in these systems is thought to cause autoimmunity in its extreme, but could small alterations contribute to autoimmune disease and T1D? That is what I have been focusing on in my work and will try to explain to you in this thesis.

The ideal immune system

The Immune system has as its purpose to safeguard us from microbial pathogens. Developed through evolution it is a fine-tuned system that can respond to an impressive number of threats. However, viruses, bacteria and other pathogens have also evolved and a constant battle between human and pathogen survival is still taking place.

Parallel to the growth of a baby, the immune system is in the beginning also dependent on the mother. Trough the umbilical cord and the mother's milk, the baby's immune system is provided with antibodies and immune complexes. As the child and the immune system develop, both are trained to respond to threats from the environment. During the first two years of a child's life, the immune system develops into what it is during the rest of the life.

The Immune system is directed by the T-cells. Using an array of cytokines, chemokines and cell surface receptors the T cells can command other types of cells and direct the most suitable response to a pathogen.

The T-cells of the Immune system are seen in many cases as the directors of the Immune system. Trained in the thymus gland in early childhood the cells are selected based on their T cell receptor (TCR) specificity by two processes (reviewed in [3, 4]). Positive selection in the cortex part of the thymus acts to ensure that the best clones of T-cells survives. In contrast, weaker binding cells are eliminated. The selected cells are submitted to negative selection in the thymic medulla where cells that react to presented autologous (self) antigens are destroyed. Ribonucleic acid (RNA) from a great variety of self-antigens, is expressed in the thymus medulla [5] which probably reflects this selection process. The autoimmune regulator (AIRE) protein seems to be an essential part of this process. AIRE gene Knock-Out mice develop several autoimmune diseases, probably due to a reduced expression of antigen on medullary epithelial cells, disturbing negative selection [6]. Failure to delete potentially autoreactive cells in this central tolerance process would in most cases lead to autoimmunity.

Autoreactive T cells do however to some extent escape this selection and are present in healthy individuals [7]. Polyreactive antibodies with affinity to autologus peptides are known to naturally exist also in healthy individuals, possibly as a early defence against microbes or as part of an elimination mechanism of worn out or malignant cells [8, 9]. To prevent that

autoimmunity develops the human immune system has a second line of defence, peripheral tolerance [1, 10].

The Immunological Synapse

The Immune system must swiftly and effectively identity threats. The natural course of a microbe after entering the system is that it is killed by innate immune system mechanisms and later phagocytised by Antigen Presenting Cells (APC). These APC are the link to the adaptive immune system and form what is often referred to as the "Immunological synapse" with T-cells.

The APC digest the microbe protein into peptides in lysosomes. The digested peptides are then merged with Human Leukocyte Class II (HLA) molecules and later expressed on the APC surface and presented to T helper (Th) CD4+ cells [54]. A Th-cell with TCR specific recognition of the presented peptide antigen will bind. This recognition is the first step of the Immunological synapse signal. What is then needed is an affirmation, the co-stimulatory signal.

When the HLA Class II – TCR CD3 complex is formed, co-stimulatory signals are needed for the signal to initiate. Surface-bound CD28 on the Th-cell till seek to engage B7 (CD80/86)-molecules on the APC and support the activation signal [11].

At the same time as this costimulatory complex is being formed, intracellular Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4, CD152) protein is activated and upregulated on the T cell surface [12]. CTLA-4 can also bind to B7-molucules, and has an even greater binding affininity for B7 than the competing CD28 [13, 14]. CTLA-4/B7 binding initiate a negative feedback signal that can stop the activation signal [12, 15, 16]. This means that an competition between activation and regulation is formed, and only the strongest activations result in a positive immune signals in the immunological synapse.

When all activation criteria are met, the Th cell directs the immune system into the most efficient course of action for the microbe that has been identified. Depending on local cytokine signal environment, the sort of TCR activation, costimulation and the antigen presented, the Th cell develops into different phenotypes [17, 18].

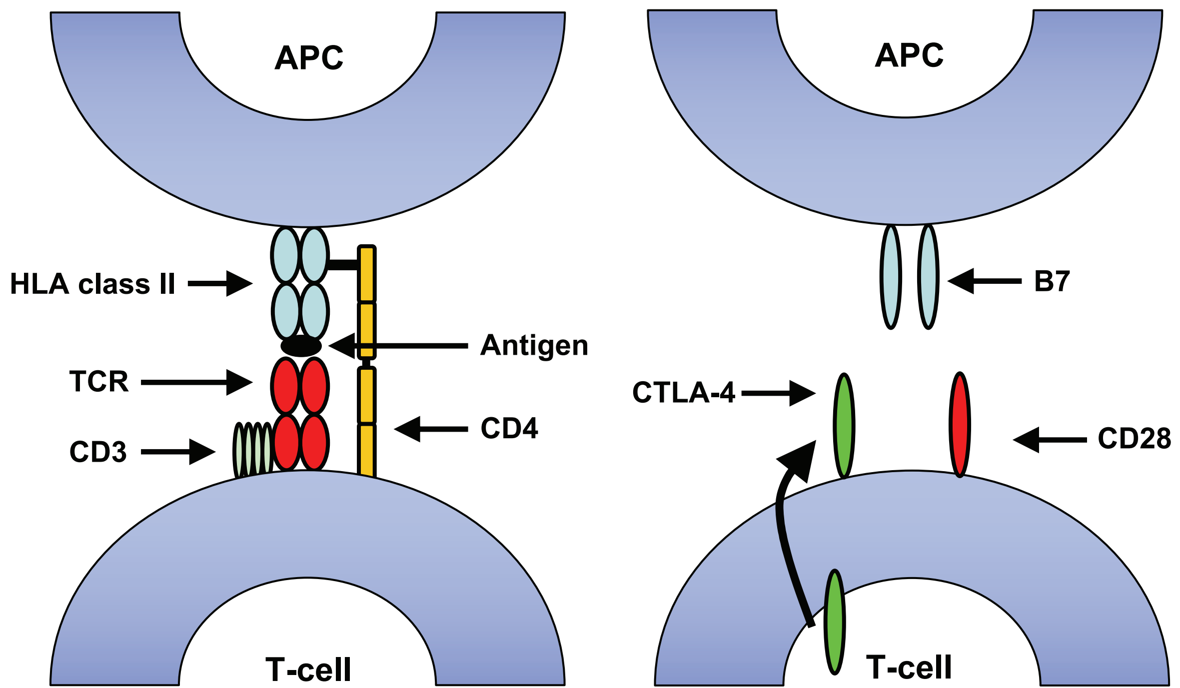

Figure 1. The immunological synapse. Activation signal (left) and costimulatory signal (right) are both needed for a immune response to take place

The Th1/Th2 paradigm and Th17

Th cells can roughly be divided into two main phenotypes based on their response, Th1 and Th2. Simplified Th1 cells promote intracellular immunity, and Th2 cells extracellular immunity. Th1 cells produce high levels of IL-2, IFN-γ, lymphotoxin α and are dependent on the transcription factor T-BET [19-21]. Th2 cells generally produce high levels of IL-4, IL-5, IL-9, IL-13 and are dependent on the transcription factor GATA-3 [19-21]. IL-12 promotes Th1 cells and IL-4 Th2 and there is a mechanism of cross-regulation that promotes a polarized response [17]. Both IL-4 and IFN-γ has been shown to suppress the recently discovered Th17 cells. Th17 cells are belived to act in immunity to extracellular bacteria and fungi, but have been implicated in several inflammatory autoimmune diseases [21].

Regulatory T cells

Regulatory T cells regulate the immune system by inducing or suppressing immune mediators to achieve immunological tolerance. The presence of suppressing cell population was suggested already in the late 1960, but it was not until quite recently this population and function became well known [21]. Regulatory T-cells (Treg) constitute the subpopulation of T-cells responsible for suppressing autoimmunity in the peripheral immune system [22, 23]. The subpopulation believed to be the most important express the cell-surface markers CD4 together with high expression of CD25 (IL-2-receptor α chain) $CD25^{high}$, which makes it possible to distinguish them from ordinary T-cells which do not express $CD25^{high}$ spontaneously [24-25].

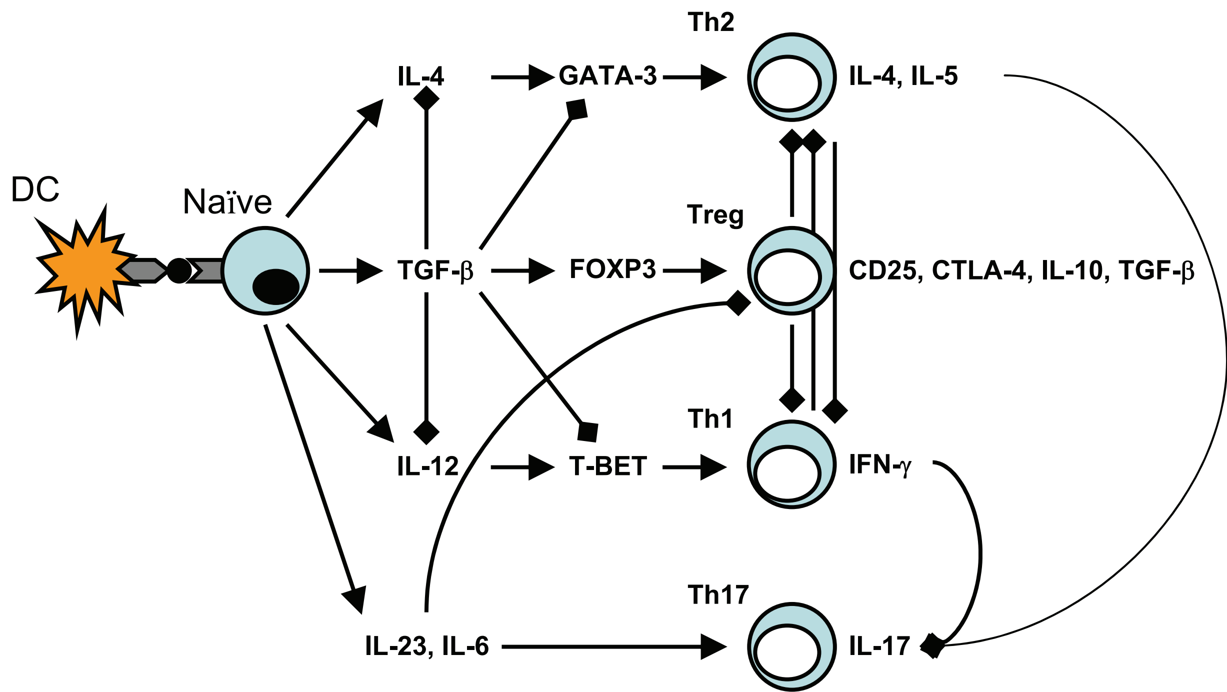

Figure 2. The Th cell linage commitmnent and network. Arrows represent stimulation pathways, and blocks inhibitory pathways.

FOXP3 has been suggested and still remains the most specific marker for induction of and identification of Treg cells [26-29]. However, recently it was found that not even FOXP3 expression can be routinely used to safely identify Treg cells since transient expression can be found in non-regulatory CD4+ T cells [30].

The thymus is crucial for the training of regulatory $CD4^{+}CD25^{+}$ cells as neonatal thymectomy results in various organ-specific autoimmune diseases in mice as a result of loss of $CD4^{+}CD25^{+}$ cells [31]. Mice depleted of $CD4^{+}CD25^{+}$ cells develop similar symptoms of autoimmunity, like infiltration of lymphocytes, insulitis and destruction of internal organs [24]. More, Treg cells that are developed in the mouse thymus have been shown to acquire both anergic and suppressive properties [32]. It has been suggested that the development of this cell population is dependent of major histocompability complex (MHC) class II-positive thymic epithelium [33]. Recent results indicate that the $CD4^{+}CD25^{+}$ cells are produced by thymic use of a special TCR-MHC affinity, different from that engaged in $CD4^{+}CD25^{-}$ thymocyte development, and that B-cells may control the pool of the migrated cells [34]. Human diseases such as T1D have been associated with a defective $CD4^{+}CD25^{high}$ population [35-37].

CTLA-4 plays a key role in the $CD4^{+}CD25^{high}$ T cell-mediated control of auto reactive T cells [38, 39]. Partly this is supported by the finding that IL-2 related immunosuppression is believed to stimulate CTLA-4 expression [40]. CTLA-4 is co-expressed with CD28, CD25 and CD45RO, and has been reported to be expressed in higher levels on $CD4^{+}CD25^{high}$ cells than their $CD4^{+}CD25^{-}$ counterparts [14, 41]. The CTLA-4 molecule is also expressed on $CD8^{+}$ cells [40].

It is not completely understood how Treg cells propagate their effect, but the explanation might be found in the molecular interaction between antigen presenting dendritic cells (DC) and T-cells. $Foxp3^{+}$ Treg cells have been shown to initiate a CTLA-4 driven tryptophan catabolism in the DC that results in regulatory capability [42]. This is supported by the earlier finding that CTLA-4 is involved in DC-mediated tolerance by tryptophan activation [43, 44]. Treg CTLA-4 or soluble CTLA-4 (sCTLA-4) induction of the tryptophan catabolising enzyme indoeamine 2,3 dioxygenase (IDO) in DC, make these cells capable of suppressing T-cell activation by way of neighbouring stimulatory DC [45].

IL-2 has been shown to be essential for the TGF-β induction of native CD4+CD25+ cells differentiation to Foxp3+ Treg cells [46]. A $CD25^{low}$-expressing population of $Foxp3^{+}$ adaptive regulatory cells has been recorded to successfully suppress T cell immunity in a TGF-β-dependent manner in diabetes-prone mice [47]. Furthermore the authors also argue that the adaptive Treg cells can be induced by anti-CD3 immunotherapy that promotes the restoration of self-tolerance.

TGF-β clearly is part of the Treg generation and expression of Foxp3 [48]. Retrovirus-induction of Foxp3 in NOD mice has been shown to generate antigen-specific T1D protective, TGF-β secreting, T-cells [49]

Surprisingly it has been reported that IFN-γ is essential for the development of Treg cells in mice [50]. Th2 like cells are less sensitive to Treg suppression than Th1 since they can stimulate themselves with IL-4 and IL-9 and don not rely as much on IL-2 for their survival [51]. Possibly this can explain why Treg are so important in Th1-associated autoimmunity. Th2 is regarded as protection in autoimmunity and another factor that might influence, is that IL-4 and IL-3 seem to be able to induce Foxp3 Treg cells even from $CD25^{-}$ precursor cells [52]. More, CTLA-4 induced signals on activated CD8 T cells reduce IFN-γ cytokine

expression by regulating the high-secreting cells and may thus prevent Th1 mediated autoimmunity [53].

The HLA system

The human HLA system, corresponding to the mouse Major histocompatibility system (MHC) system is a fundamental part of the immune system in all animals. It was first discovered in transplant reactions (histocompatibility) where tissue from non self is rejected (graft reaction) if an incompatibility arises [54]. The purpose of this system is to act as identifying markers on all the body's cells. HLA class I identifies cells to cytotoxic T cells whereas HLA Class II is involved in antigen presentation to T helper cells.

The class II genes codes for the four subunits of the HLA Class II complex. The complex is formed by two α subunits and two β polypeptide subunits. These are coded by the D-class genes of the P, Q or R family of genes and code for a α or β subunit [54]. This is how the HLA nomenclature is formed. HLA-DQB1-0302 for example should be read: 0302 allelic variant of gene 1 coding for the β-chain of HLA Class II Q family. The structural parts $\alpha1$ and $\beta1$ make up the peptide-binding domain, $\alpha2$ and $\beta2$ the immunoglobulin-like domain and followed by the transmembrande (TM) domain and the cytoplasmic tail (Fig 3). When the HLA has been coded DQA1 and DQB1 from the same chromosome a *cis* binding is formed. When the HLA has been coded from different chromosomes it is in *trans* formation [55]. The DQ molecules are polymorphic, which means that both the A1 and B1 gene variants are used for identification. In DR molecules on the other hand just the beta-chain is polymorphic, thus needing only one identifier DRB1*0401 or shortly DR4. These genes are not inherited randomly but are in linkage disequilibrium which allows for a deductive approach in identification.

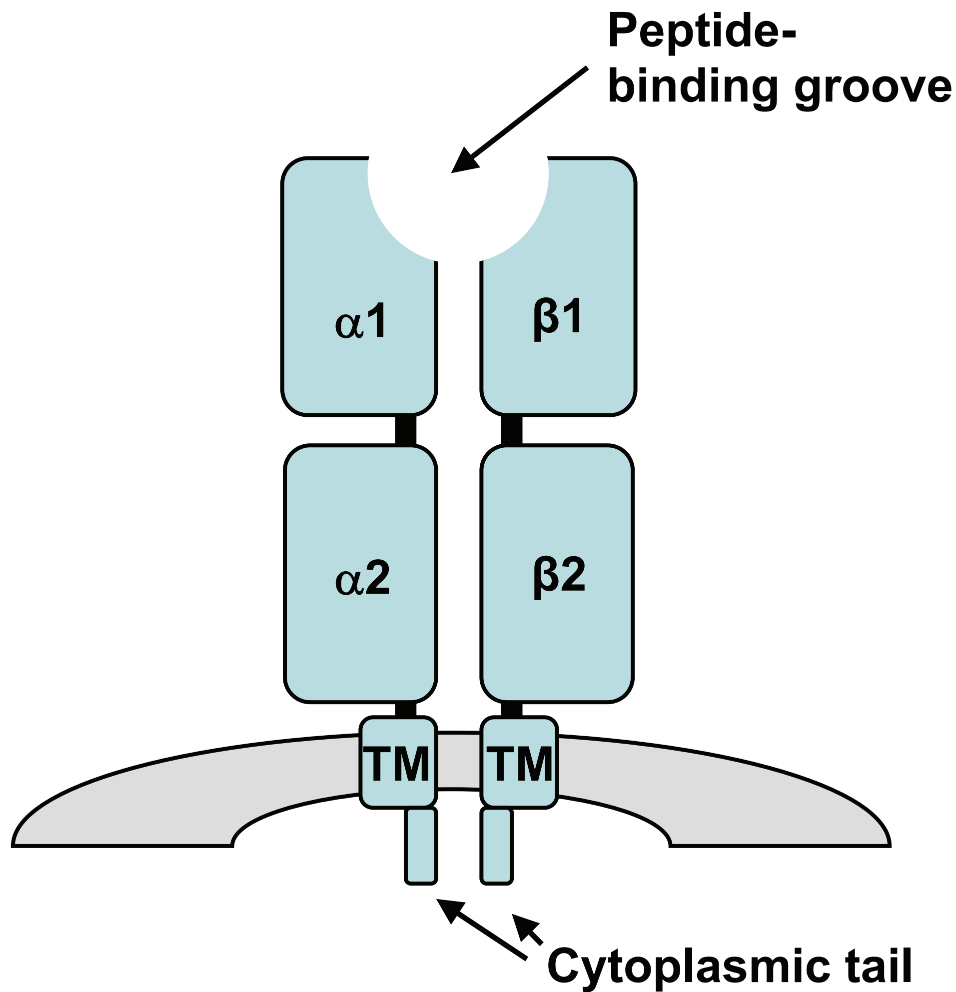

Figure 3. The HLA Class II molecule

Each set of alleles have one set inherited from the father and one from the mother. The combination of genes and the alleles from the two chromosomes makes a human able to make up an enormous variety of HLA molecules. Most likely this has played some part in evolution of the human race as different combinations may affect HLA Class II peptide presentation and resistance to diseases [56-58].

When an APC endocytise a foreign protein it is fused with an endosome and subsequently with a lysosome containing a HLA Class II molecule. As the protein is degraded the peptide-HLA Class complex is transported to the cell surface. The peptides then become available for Th cell recognition and in the case of pathogens, initiate immune system activation [54].

CTLA-4

CTLA-4 was found in a mouse strain when screening for genes in the immunoglobulin family. The gene is translated to a 223-amino acid protein that is mainly expressed in activated lymphocytes, coinduced by cytotoxic T cell activity [59]. The human gene is located on the chromosome 2q33 and shares identical regions with the murine CTLA-4 [60]. This evolutionary conservation might be due to the protein's biological importance. CTLA-4 is closely related to CD28 as to sequence, gene structure and chromosomal location in both human and mouse [13]. CTLA-4 protein is mainly expressed in endosomal compartments in T cells, but is upregulated very quickly to membrane surface when activated [12, 61].

CTLA-4 -/- mice develop a B7/CD28-dependent fatal lymphoproliferative disease without antigen stimulation [62]. It was observed that progression of this condition could be controlled by administration of CTLA-4Ig. Lymphocytic infiltration began in pancreas (and other tissues) 14 days after treatment was stopped.

T-cells, including Treg cells, upregulate cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152) from endosomal compartments, upon stimulation [12, 61]. CTLA-4 can like CD28, bind to B7-molecules [15]. At CTLA-4/B7 binding an inhibitory immune signal is activated. In animal studies it has been shown that CTLA-4 is essential to avoid a deadly lymphoproliferative syndrome [62]. Expression of CTLA-4 is associated with regulatory functions in the immune system. CTLA-4 binding can induce apoptosis in previously activated T cells, whereas freshly isolated cells are halted in cell cycle progression [10, 63]. Induction of apoptosis seems to be antigen-specific since there is a need of TCR-co-binding. It has also been established that the B7/CTLA-4 binding might affect both the APC and the T cell trough modulation of intracellular trypthophan catabolism [44].

CTLA-4 is vital for tolerance since it introduces a threshold for immune activation. When CTLA-4 is blocked by antibodies autologous antigens are sufficient to activate immunity [64]. CTLA-4 works in parallel with Treg since the absence of either one result in autoimmunity [64].

The faulty immune system

T1D share many characteristics with other diseases caused by the immune system and affected by genetic and environmental factors. Common for these autoimmune diseases is that the immune system attacks autologus peptides and destroys that target organ. Depending on which organ or tissue that is attacked, different symptoms arise. Autoimmunity can be systemic as in Systemic Lupus Erythematosus (SLE) or organ-specific as the case of T1D.

All autoimmune diseases have been suggested to be initiated by one single antigen, followed by autoimmune response to several autoantigens as the disease develops [1]. Genes may predispose individuals by affecting immunoreactivity, antigen presentation or tissue physiology (Fig. 4). Contributing to this susceptibility are environmental factors such as nutrients, microbial flora and toxins which may also alter immune responses, and in the case of microbes molecular mimicry may contribute to disease [1]. Cross-reactivity arise when for example a virus has similar sequences as peptides in a self-protein and the successful immune response towards that virus also initiate an mistaken reactivity against the self-protein and thus cause autoimmunity [65-67].

Autoreactive cells and crossreactivity are likely to be present all the time, which makes successful peripheral regulation imperative for avoiding autoimmunity. The definition of an autoimmune disease can of course be discussed but generally there is a list of criteria that at least partly need to be met. B-cell clones that produce antibodies specific for autoantigens, T-cells responsible for disease progression and the capability of transferring the disease to a new host and genetic and animal models that develop the disease [68],

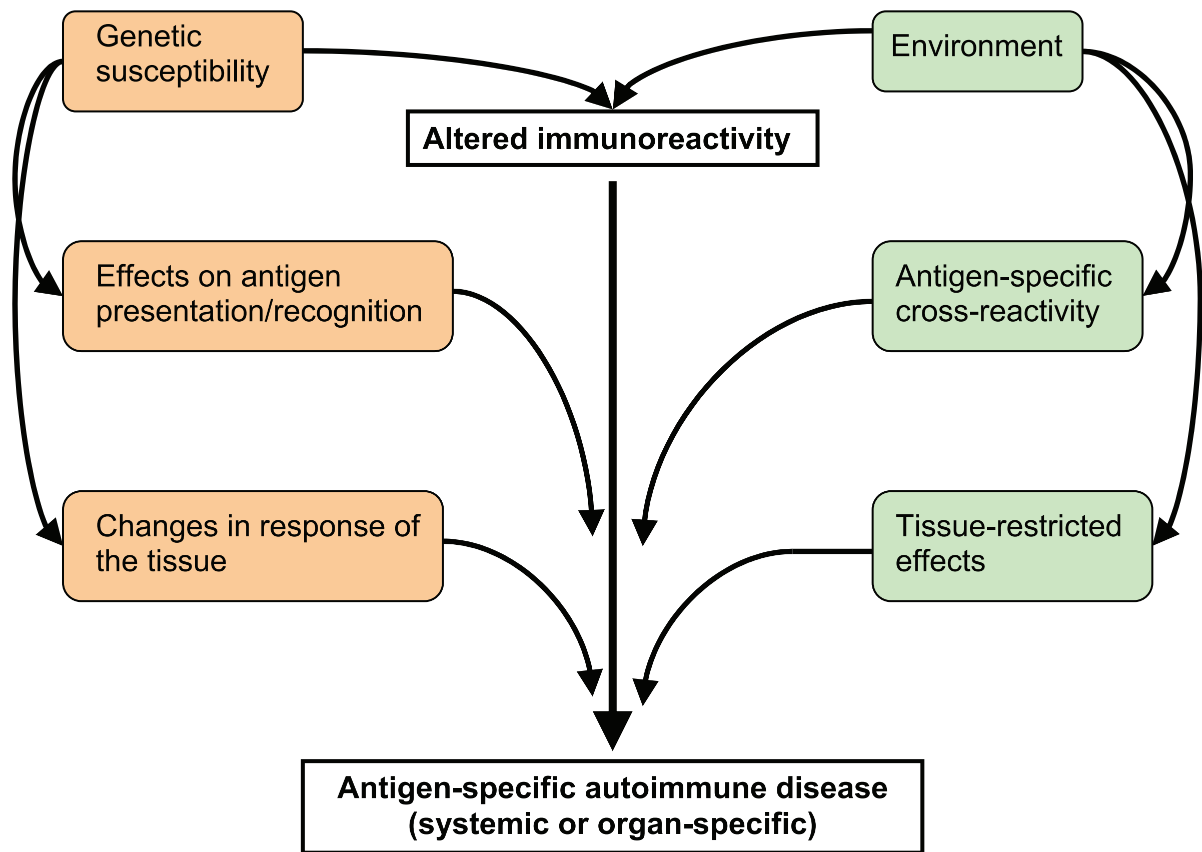

Figure 4. Pathways to autoimmune disease. Adapted from [1]

Type 1 Diabetes Pathogenesis

T1D pathogenesis progresses from a state of genetic susceptibility to diagnosed T1D by and elusive chain of events. Several clues can be found in immunological and clinical markers and many efforts to describe the course of events has been suggested in the form of models.

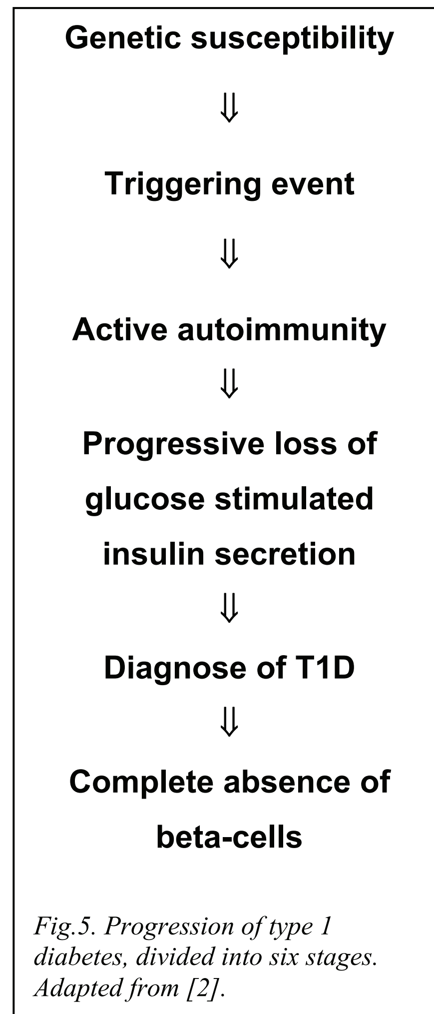

Fig.5. Progression of type 1 diabetes, divided into six stages. Adapted from [2].

Several genes are known to predispose individuals for T1D, but genetics alone fail to explain the great increase of incidence during the last decades as well as the great increase of risk in northern Europe [69-71].

T1D is caused by the autoimmune destruction of the insulin-producing beta cells in the human pancreas. Lymphoproliferation can be observed to occur prior to clinical diabetes [2]. Cells and antibodies reacting to Insulin [66, 72], Glutamic Acid Decarboxylase 65 (GAD_{65}) [73] and insulinoma-associated antigen 2 (IA-2) [2, 74] has been found. Although the mechanism is still unclear Peripherin, a neurocrine antigen that is recognised by islet-infiltrating beta cells seem to be specific for T1D at least in mice [75].

The destructive autoimmune process of beta cells may progress for several years without symptoms. It is not until about 80% of the original beta cell mass is destroyed that clinical symptoms may be discovered [2]. The diagnosis of T1D normally involves a blood glucose test, or in some cases an oral glucose tolerance test. In a healthy individual glucose is rapidly extracted from the blood to peripheral tissue by the help of induced insulin secretion from the beta cells. In case of T1D the individual's blood glucose is above a certain cut-off set by WHO. Diagnosis based on C-peptide levels can also be used. C-peptide is component of pro-insulin and is spliced of as insulin is secreted. However, since 20% of the beta cell mass may still be left at the time of diagnosis, C-peptide might not yet be zero.

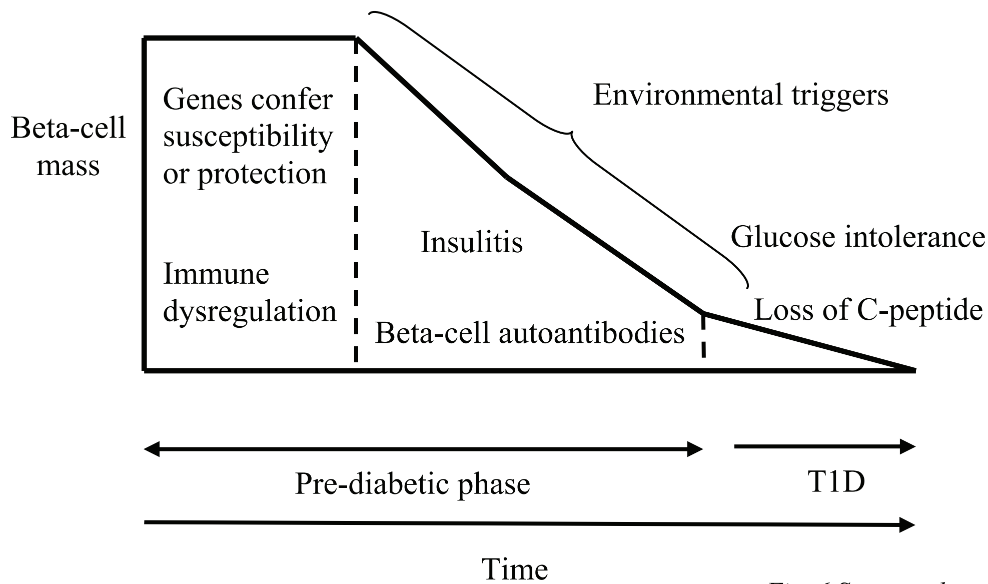

Fig. 6 Suggested natural progression of T1D, adapted from [76]

T1D meet the criteria for autoimmunity by several variables. T1D can be transferred in mice [77, 78]. There are mouse models that develop the disease, NOD mice [79] and also the BB rat [80]. T1D can adoptively be transferred between NOD mice both by CD4+ [81] and CD8+ cells [82] and can be completely avoided by administration of anti-CD4 antibodies [83].

Some viruses has similar peptide sequences as those found in the insulin producing beta cells, which suggest that the immune system could mistakenly attack the beta cells instead of virus infected cells [65, 67]. Antibodies against these intracellular beta cell proteins are present in 70-80% of newly onset T1D patients although it is no clear if this is a cause or a consequence of the attacked beta cells [84]. This kind of molecular mimicry has been observed between Coxsackie B virus and GAD_{65} protein, and is supported by antibodies and cells reactive to GAD_{65} in T1D patients [65, 67, 85].

Islet cells have been shown to process and express GAD_{65} peptide and present these peptides to T effector cells [86, 87]. More recently it was reported that a HLA Class II dependent endothelial cell transmigration of autoreactive T cells into the islet may take place, thus possibly accounting for a critical step in T1D pathogenesis [88]. Central gene activities affecting key immune functions in CD4+ cell have been observed to be down regulated in T1D [89].

Over-nutrition common in western lifestyle results in increased growth in height, body-mass index (BMI) and earlier onset of puberty. During puberty insulin resistance rise and coincides with the highest age-specific T1D incidence in both boys and girls [70]. Cow's milk has been proposed as an environmental factor affecting the incidence of type 1 diabetes [90]. It has been observed that delayed exposure to cow's milk is correlated with reduced incidence of type 1 diabetes, but the expected difference in immune response of diabetic and healthy subjects have not been seen [91].

Genetics of Type 1 Diabetes

Twin studies reveal that about 49% percent of T1D risk is based on genetic factors [92], the major susceptibility genes of T1D is located in the HLA Class II coding region. As many as 95% of T1D patients are carriers of HLA risk alleles [2]. The risk has further been pinpointed to the HLA-DQ and DR-loci [55, 93].

HLA DR3 and DR4 are associated to T1D, although they are not though to be directly pathogenic. HLA DR3 is in linkage disequilibrium with DQA1*0502-DQB1*0201. DR4 on the other hand is inherited together with DQA1*0301-DQB1*0302 [94]. HLA DR3-DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) and/or DR4-DQ8 (DRB1*04-DQA1*0301-DQB1*0302) haplotypes correlate with T1D in multiple ethnicities [2, 93, 95]. The DR15(2)-DQ6 (DRB1*15-DQA1*0102-DQB1*0602) however, has been shown to protect from T1D although predisposing for Multiple Sclerosis [96].

The HLA class II antigens DR3 (DRB1*0301) and DR4 (DRB1*0401) are present in ninety-five percent of patients with T1D [2]. HLA DQA1*0501- DQB1*0201 and DQA1*0301-DQB1*0302 show a strong association with autoimmune diseases, such as type 1 diabetes (T1D) and celiac disease [93, 95]. High HLA risk can be identified in the presence of HLR DR3 DR4 and extreme risk can be identified if the sibling to a T1D child share the same two inherited HLA haplotypes with their sibling, suggesting that there are more HLA Class Risk alleles in the vicinity of HLA DR3 and DR4 [97]. DQA1-alles can satisfyingly be deduced from the DQB1-allele typing and thus be used for T1D risk prediction [98, 99].

Diabetes Risk*	HLA DQB1 Genotype			
	Haplo 1	Haplo 2	n	Will Develop Diabetes
Very high	02†	0302	616	1/15
High	0302	0604	207	1/20
High	0302	X	1375	1/30
Moderate	02†	0604	232	1/40
Moderate	02†	X	1151	1/75
Neutral	02††	0604	126	1/500
Neutral	02††	X	641	1/500
Neutral	02††	02†	431	1/500
Neutral	02††	0302	322	1/500
Neutral	0301	02	1114	1/500
Neutral	0301	0302	750	1/500
Neutral	0604	X	397	1/500
Neutral	X	X	594	1/500
Low	0301	0604	272	1/750
Low	0301	X	1744	1/750
Very Low	0603	0302	353	1/750
Very Low	0603	02	518	1/750
Very Low	0603	0301	364	1/750
Very Low	0602/0603/0604†††			1/750
No risk	0602	0302	722	1/1000
No risk	0602	02	1005	1/1000
No risk	0602	0301	834	1/1000
No risk	0602	X	1553	1/1000

Table 1. T1D risk as assessed by HLA DQB1 hapolotypes in TEDDY clinical trial [99].

There are four known polymorphisms in the CTLA-4 gene. The microsattelite (AT)n repeat (>86bp) in the 3´ untranslated region (UTR), a single nucleotide polymorphism (SNP) in the promoter region (-318 C/T) and a SNP in exon 1 (+49 A/G) [100, 101]. More recently, CT60-polymorhpism was also found [102]. The four polymorphisms are related to autoimmune diseases and to each other [102-104]. The microsatellite assay has been proposed as less reliable than the SNP's since there are many alleles, and the alleles are amplified unevenly [105]. Several studies have confirmed the correlation between CTLA-4 +49GG alles and

diabetes [101, 106, 107]. The CTLA-4 +49A/G polymorphism has been shown to result in incomplete glycosylation of the protein and a lower cell surface upregulation [108]. The polymorphism results in an amino acid change from Thr to Ala which could influence the signal peptide of CTLA-4 that it is coding for [109].

Recently a new important susceptibility gene was identified in T1D. Inositol 1,4,5-Triphosphate Receptor 3, shows considerable risk in risk allele carriers [110]. ITPR3 is considered to influence energy metabolism and cell growth [111, 112]. Lymphoid protein tyrosine phosphatise (LYP) PTPN22 risk gene has been associated to T1D and could be involved in TCR signal modulation [113]. PTPN22 risk variant has been suggested to be involved in insulin autoantibody antibody formation and the progression to T1D [114]. Insulin variable number tandem repeats, INS VNTR has also been implicated in T1D predisposition and might affect autoimmune target specificity or thymic expression of insulin [115, 116].

Epidemiology of Type 1 Diabetes

Diabetes is a common disease in western countries with a peak incidence in northern Europe. Sweden has the highest incidence of type 1 diabetes next after Finland, whereas Japan has the lowest incidence in the world (1-2 per 100 000 and year) [69, 117]. The incidence in Sweden at age 0-34 between 1983 and 1998 was 21.4 men and 17.1 women per 100 000 a year [70]. The age of onset tend to be decreasing and might at least partly explain the increasing incidence. Type 1 diabetes is more common in male patients than females. This is unique for diabetes, since all other organ-specific autoimmune diseases show a female bias [118]. Type 1 diabetic fathers also transmit the disease to a larger extent, than mothers [118].

Eight percent of T1D first degree relatives have autoimmune thyroiditis, and about 10 percent have Celiac Disease (CD) [119, 120]. It is possible that HLA risk allele accumulation in northern Europe is a result of natural selection towards disease resistance. It has been suggested that Rheumatoid Arthritis incidence increase is caused by selection of Tuberculosis (TB) resistance since Tumor Necrosis Factor (TNF)-α that is important in TB immunity is more pronounced in individuals that have RA susceptibility genes [121]. Another intriguing theory is that the DR3 and DR4 are descendant from two distinct populations, and that when they meet (as in Scandinavia) it results in a unfortunate high prevalence of T1D [122].

The patient

Having a chronic disease with life threatening complications can be very hard for a child or adolescent. Having to cope with the constant worry of correct insulin dose or nutrition consumption at the same time as you are in a phase of your life when independence is important can of course cause many conflicts.

Although T1D is a disease where the individual has lost the capability to harvest the glucose from the blood, the acute risk in an individual's day to day life is hypoglycaemia. By administrating long-lasting insulin, eating a balanced diet, monitoring blood glucose and patient education the risk of hypoglycaemia can be reduced. However, in a case where the equilibrium of glucose metabolism, insulin dose and food is disturbed, life threatening hypoglycaemia can arise. In these cases, it is very important that surrounding witnesses; family, friends or school staff quickly recognise the symptoms and administer glucose or equivalent. In cases where it is very hard to set the insulin dose the patient may get an insulin pump. The pump is seen as a small bag in the belt with tubes entering the stomach. This device monitors the blood glucose and can administer insulin at a more physiological manner.

T1D patients have to go to the doctor all their life. In the beginning of the disease, education and finding a correct dose of insulin are primary.

Hypothesis and aims of the thesis

Our aim was to investigate the effect and role of CTLA-4 and HLA Class II in the T1D immunity. We hypothesised that much knowledge could be gained by studying primarily healthy children, and comparing with T1D patients. By focusing on the immune responses associated to T1D in healthy children with risk genotypes we aimed to unravel some of the mechanisms of which these genes confer T1D risk.

The specific aims and hypotheses were:

I. To investigate interactions of CTLA-4 +49A/G and HLA Class II Risk genes in T1D-associated immune responses. Our hypothesis was that these genes could potentially affect Th1/Th2 like immune responses to T1D antigens.

II. To explore CTLA-4 and HLA Class II polymorphism effect on the regulatory T cell population in healthy children. We hypothesised that the risk alleles of these genes could affect the number or phenotype of the Treg population that so important for maintaining peripheral tolerance.

III. To follow up on results from paper I. We aimed to investigate if risk-associated effects from the previously studied genes could be observed even after T1D was diagnosed, and how a T1D and healthy population are related.

IV. To apply our previous study design to scrutinise the results from a clinical intervention trail of T1D. We hypothesised that although very limited clinical outcome of the study, Treg-associated immune modulation was achieved.

Subjects & Methods

The ABIS study

ABIS, short for All Babies in Southeast Sweden (Alla Barn i Sydöstra Sverige) is a study initiated by Professor Johnny Ludvigsson at the Division of Pediatrics and Diabetes Research Centre at the Linköping University.

ABIS was originally designed as a prospective cohort study to study the presence of type 1 diabetes, autoimmune diseases and allergy in the general population. 17.000 families were enrolled out of the invited 21.700 families giving birth between October 1st 1997 and October 1st 1999. Blood, urine, stool and hair samples were collected in an ambitious protocol following the children up to five years of age [123]. The constant supply of fresh blood samples from an unselected child population has made it able to conduct many studies; one among them is Paper II were we were able to isolated PBMC from fresh blood.

The reason that these children were used is of course affected by the availability. However, this age group and somewhat older is a good population to study. The children are old enough to have a mature immune system and in an age where T1D is possible. They are also young enough to not be affected by puberty. Although puberty is a possible accelerator of T1D the hormones could influence the immune system in a way that would make it harder to dissect the results.

Healthy School Children

School healthcare collected samples from 70 healthy children in the ages from 7 to 15 years old. In order to even out the distribution of collected samples a maximum of 3 boys, and 3 girls volunteering children from each class were selected. All children were from the same school. The children were asked to fill out a form together with their parents with questions about their own and theirs family's health. An informed consent was also collected from participants and their parents. Children excluded from the study had an ongoing infection or were atopic, diabetic or had these or other autoimmune diseases in close family.

T1D Diabetes patients

Blood samples from 30 children (3 to 17 years of age, average 10 years of age, 14 female (F)/16 male (M)) diagnosed with T1D were collected at the pediatric clinic at the Linköping University Hospital. Samples were taken at three different occasions 0-3 (sample (S1), 10-18 (S2) and 20-48 (S3) months after diagnosis. Patients were asked to give one additional blood sample, for research purposes, at regular visits to the clinic. Informed consent was given by both the parents and the child.

T1D children enrolled in Photopheresis intervention trail

Twenty children from a previously performed randomised double blind placebo controlled trial (described in detail elsewhere [124]) were selected for a follow-up study on immune modulation. Patients with recent onset (5-6 days post diagnosis) T1D (10-17 years old) were enrolled in the original study and allocated to receive active or placebo extracorporeal photochemotherapy (ECP). Ten patients were actively treated and ten patients matched for age and gender, were placebo treated. Active treatment consisted of an oral dose of 8-methoxypsoralen (MOP) two times 0.6 mg/kg and ECP procedure where buffy coat cells were irradiated with 2 J/cm^2 UVA light for 90 minutes after which the cells were returned to the patient's circulation. The treatment was repeated two times on two consecutive days. Placebo treatment consisted of placebo tablet and apheresis treatment. The first treatment session was done approximately 5 days after T1D diagnosis, and the subsequent treatments at 14, 28, 42 and 90 day's duration. Peripheral blood was taken before each treatment session. PBMC from these samples were stored in liquid nitrogen and available for follow-up studies.

Since this was a clinical trail ethical considerations were important. This project utilized surplus samples from an already conducted ECP-trial. The original trial was ethically considered with the arguments that ECP could be performed with minimal discomfort and risk for adverse effects and that the benefits of a positive result could be enormous for test subjects as well as other patients. We saw no major ethical problems extending the original study and further analysing the already taken samples, as allowed as part of the original ethical approval.

Flow cytometry

Flow cytometry is a method to identify, isolate a number of cell types and their activity [125]. A two-laser system flow cytometer makes it possible to divide the light in to four different ranges of wavelengths (channels), each specific for different flourochromes. These flourochromes are conjugated to antibodies specific for different (immune) markers. The four-channel model makes it possible to study four different markers at the same time on each cell, when passing through the lasers in the flow cytometer. Common flourochromes are Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin-Chlorophyll-Protein (PerCP) and Allophycocyanin (APC). The different ranges of excited wavelengths of each flourochrome overlap each other to some extent thus making software compensation necessary. A flourochrome-conjugated antibody will emit light when hit by the right wavelength. This light-signal is recorded and fed into the FACS software. All the signals of a cell are displayed on a dot-plot in the graphical interface of the FACS-software. The intensity of the signal of each channel corresponds to the amount of the immune marker and, the stronger the signal, the further out on that plot's and dedicated wavelength-axis the dot is made. The plots are limited in two dimensions which makes it necessary to use several plots in order to compare each channel against each one of the other channels.

Cells from one individual can be stained in different combinations and is aliquoted into different tubes, generating data collected into computer files. These files can later be analysed in analysing software. With the use of gates set in different plots, and using the data from one gate in another plot, it is possible to export selected data and observe more than two markers at the same time. Another use of gating is to set at "–high/bright" expression in plots with a certain channel, and analyse the gated data in another plot with respect to other makers. This method needs much consideration, since it might be criticised as arbitrary. The output of the analysis is the amount and percentage of cells occurring in each quadrant of a plot, e.g. "channel 1- Channel- 3 double positive". Additionally, in the case you are certain that you're not interested in a specific cell you can do a negative selection of the marker specific for that cells. The resulting figures can later be fed into a statistics program and compared to samples from other subjects.

Analysis of figures can be done in several ways, depending on your interest of outcome [126]. The number of markers expressed on each cell in your selected (sub)population of cells can be

derived from the Mean Fluorescent Intensity (MFI). Another perspective on the outcome is to identify percentages of certain subpopulations of cell, in relation to the total population of interest.

As can be easily comprehended, flow cytometry is a method that can generate a great yield of data from just one sample. The gate setting is an important step, and there is a risk of subjective variation. Because of this the analysing should be done by one or few people trained in consistency. Flow cytometry is a good method to characterise cells, provided you have specific markers for your cell of interest and antibodies directed against these.

ELISPOT

Enzyme linked-immunospot assay is a very sensitive assay for enumeration of single cells secreting as certain immune marker, a cytokine [127]. The assay has a wide array of applications but is best suited to measure specific response of a low percentage of cells and by an elusive cytokine. This is particularly useful in the field of autoimmunity research since a very low number of autoreactive cells might still be interesting [7].

You start of with a 96-well plate microtiter plate with a bottom made out of PVDF or nitrocellulose membrane. The PVDF membrane is hydrophobic and needs to be treated with ethanol to become hydrophilic for the following steps in the assay. After the short exposure to ethanol the plate is washed with water several times in order to remove any ethanol left. The membrane is now prepared for coating with a high affinity monoclonal antibody directed to the cytokine of interest. It is of course possible to divide the plate in different sections and coat with different antibodies. One common procedure is to divide the plate into IFN-γ and IL-4 detection areas, to measure Th1 and Th2-like cytokine responses. Here the protocol allows for a rapid coating procedure in humid 37°C 5% CO_2 environment, or humid 4°C over-night coating incubation. Antibodies are added in a high concentration to ensure maximum detection capability. Excess antibodies are washed away after which Iscove's or other culture medium with added Fetal Calf Sera or Human Sera is used to block any gaps in the antibody cover of the well. Fresh or frozen selected or unselected cells up to an amount of 250 000 per well (normally 100 000) are then added in the presence of an antigen. Generally, you study the cytokine responses to a set of antigens, to unstimulated cells (negative control) and mitogen (positive control). As positive control phytohemaglutinin (PHA), tetanus toxin or CEF (mixture of antigens derived from pathogens most are immune to) can be used. PHA can also be used to measure immune system responsiveness, but the great number of spots might require a lower number of cells in those wells in order to be easily quantified. The use of scrambler protein/peptide antigens or irrelevant antigens can be used to further support the specificity of the findings. Preferable sample cell stimulations should be done in triplicates or quadruplicates. The wells in the outer rim of the plate should not be used since differences have been observed in these wells, probably due to circulation of moisture.

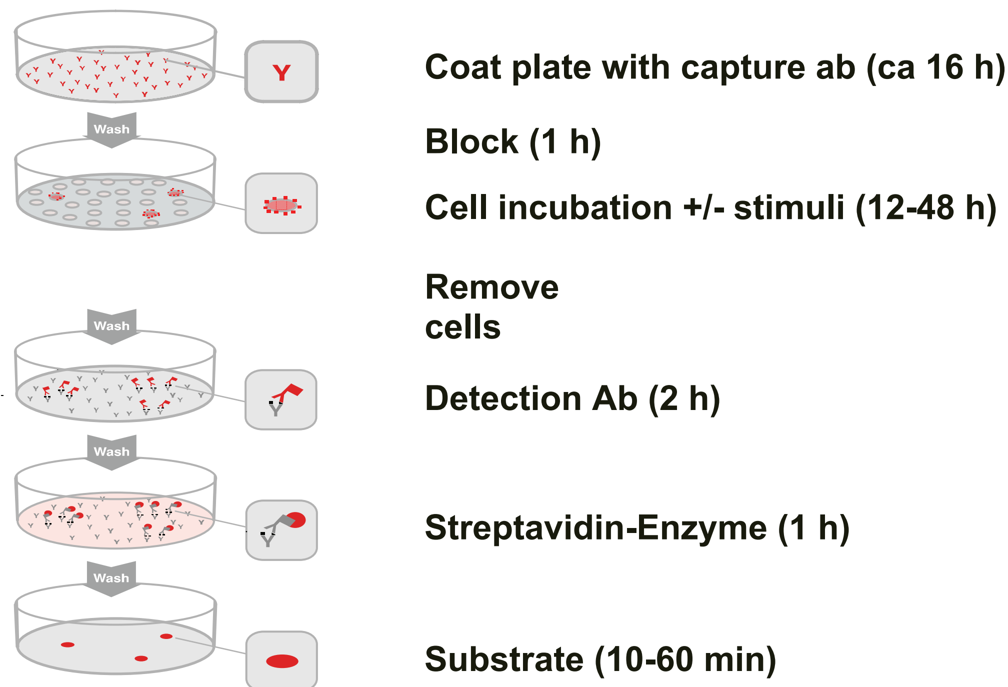

Figure 7. Standard protocol for Enzyme-linked ImmunoSPOT Assay (ELISPOT)
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The plate is then incubated in a humidity chamber at 37°C 5% CO_2 48 hours, or the optimized time for the antigen and cytokine of interest. Spot development start with the removal of cells from the plate by quite forcefully shaking and slamming out any liquid on a small stack of paper towels. The plate is the exhaustively washed to prepare for the addition of biotinylated monoclonal antibody again directed against the cytokine of interest. The antibody will find and bind to the cytokine that has been captured by the coating antibody as the cell has secreted that specific antigen. One advantages of ELISPOT is that the cytokine is directly captured by the coating antibody and can not be digested or metabolised as for example IL-4 often is in enzyme-linked immunosorbent assay (ELISA). Excess biotinylated antibody is then washed away before Streptavidin-Alcalic Phosphatase (AP) is added. The Streptavidin binds to the biotin-part of the capture antibody and prepare for the step that develops the spot. After incubation and washing a colour development buffer is added containing substrate for the AP. The product of the enzymatic reaction is a dark blue dye that will stain the PVDF membrane where the capture antibody is located, thus where the secreting cell has been. The result is a “shadow” of each cell that has secreted the cytokine coating and capture antibodies were directed against. The plate is rinsed and dried and can be stored as the bottom PVDF “paper strip” until spot count.

Spots can be counted manually although it is a tiresome and time demanding job. In order to speed spot counting up several ELISPOT readers are available on the market. Except saving time these readers also reduce the risk of counting subjectivity. The readers works as a digital cameras that photograph each well. A software program is then used to mark spots based on size colour intensity and blurring around the edges. A manual confirmation of the reader's results is however necessary, since any contaminating artefacts can in some cases be incorrectly registered as spots. This manual part of course introduces a degree of subjectivity which makes a run-in period necessary. After some time subjective confirmation is very consistent.

After-assay data processing involves positive and negative control approval and summary of the quadruplicate samples. Unstimulated secretion count is subtracted from antigen-stimulated responses in order to acquire antigen-specific spot figures.

Altogether ELISPOT is a time-consuming assay but has great sensitivity and can be used to measure antigen-specific responses as well as altered responsiveness after intervention. Compared to ELISA you here work without dilution effects and with a direct capture of secreted cytokine. Another advantage is that the assay shows you directly what happens and depending on if you select your cells you have a very express view of your *in vitro* cell responses. The most tiresome and subjective step - the counting - can possibly be avoided when new Flourospot assay and readers become available. The spots are formed by fluorescent dye that can be automatically read in a reader adapted for that purpose.

Real-Time PCR

Real Time quantitative polymerase chain reaction (PCR) is a sensitive method of measuring messenger RNA (mRNA) in cells. The method can also be used for genotyping and is sometimes used in clinical medicine to detect the presence of pathogenic DNA or RNA in patient samples. The method relies on the use of Polymerase Chain Reaction to specifically amplify target DNA.

In the case of blood, sera or cells the first step in the protocol is to isolate the RNA. This is done by a series of steps in RNA isolation columns. Cells are mixed with a solution that lyses the cell membrane and inactivate RNAses that otherwise could consume the intracellular RNA. The lysate is spun in a filtration column that removes the pieces of waste and DNA as the liquid passes trough. The resulting filtrate is then added to a new filter column that is designed to bind total RNA. The filter is then moved to a collecting column. RNAse-free double distilled water is added to elute the RNA in the filter, and the sample is collected at the bottom of the tube as the column is spun.

The resulting collection of total RNA needs to be transcribed to prepare it for Real Time PCR. Before that, there is an optional step to eliminate any traces of genomic DNA by means of DNAse enzyme. The enzyme is sometimes argued to be somewhat unspecific and thus lowering the concentration of RNA in the sample. Complementary DNA (cDNA) synthesis from the RNA template is then done with a kit containing the vital Reverse transcriptase enzyme, oligo (dT)'s to specifically initiate transcription of mRNA poly-A-tail, dNTP's as building blocks and chemicals to support the reaction. The reverse transcriptase mix is run at a temperature optimal for the enzyme, which is then inactivated by a 95°C step at the end of the program.

A combination of primers and probes are used to measure the transcription and quantify the original amount of mRNA in the sample. The primers are used to initiate transcription of a single-stranded DNA at each side of the area of interest. A probe with a fluorescent reporter dye attached in one end and a quencher in the other end bind specifically somewhere along the targeted sequence. The reporter and quencher are aligned so that the reporter does not emit any fluorescence as long as the probe is intact. When the polymerase reaches the site of the probe, the reporter dye is bumped off from the DNA. Now that the probe is no longer intact,

the reporter dye will emit a fluorescent signal that is recorded by the qPCR machine. In the cycling PCR sequence a probe is joined to the target DNA in every cycle, and one signal is thus emitted for every time the DNA is replicated. As every DNA strand give rise to two new ones in every cycle, the amount of target DNA is growing exponentially, as do the emitted reporter signal.

At the first cycles the reporter signal is to low to be detected, but it can be recorded in just a few cycles. This action takes place in all sample wells, and depending on which primer/probe signal has been selected and the original concentration of the DNA the recorded fluorescence is at different intensities at the same number of cycles. This is something that is used in the later analysis of the qPCR reaction. When the program has come to halt, fluorescence is plotted in a diagram that is used for data processing. A cut-off value is selected based on a line when all amplification plots are in an exponential increase (log-linear) phase. The analysis software then measures how many cycles each well/sample needs to reach that cut-off value. This can be translated to an estimate of how much the original concentration was of mRNA. However, to isolate the data from variables that could influence the results data is calculated as relative to controlled variables.

The data procession of this last step is however a matter of debate. The Cycle threshold (Ct) is an arbitrary value and is dependent on several factors that might influence the output. One vital step is to relate the Ct to the amount of cells in the starting material. This us usually done by selecting a housekeeping gene as an endogenous control that is insensitive to stimulation and represent the cell. The target Ct is related to the endogenous control Ct. The endogenous control can be run in the same well by using another reporter dye (multiplex) if there is no interference of the qPCR reaction. Next, one option is to use a standard curve on each target gene based on one sample. The adjusted target Ct is then plotted in the standard curve and the expression of target mRNA can be expressed as Arbitrary Units (AU). One concern with this method is that it consumes a lot of space on the qPCR plate, and might force you to run a sample separated by two plates, if you have a number of mRNA signals you want to study. Another option is to relate all samples against a calibration sample that is run on all plates. In this manner you certify that the inter-assay variability is not too big and adjust your data to one standard. You then calculate the relative transcription by this formula:

Δ Ct = Ct Target – Ct endogenous control (data normalised for amount of cells)

$\Delta \Delta$ Ct = Δ Ct – Ct Target calibration sample (data normalised for plate)

Relative transcription = $2^{\wedge(-\Delta\Delta Ct)}$ (linear data normalised to transcription of target gene)

You may also add one step where the Target Ct is related to an unstimulated sample if your protocol involves stimulated samples.

One important aspect of this formula is that it assumes perfect exponential amplification, which makes it imperative to make sure that the combination of primers and probes in your samples are amplified in this way.

Real-Time qPCR is a highly sensitive assay to measure mRNA. However since the assay is that sensitive, good laboratory manners are important. Even very small traces of contamination may affect your samples. One way of avoiding this is to change gloves often and run any material that goes into the flow cabinet in a Stratalinker, which is designed to break any RNA or DNA. Avoid working directly over your samples, especially with clothes. Skin fragments contain RNAse enzymes that can destroy your samples. Your primers and probes must be very specific, preferably they should be designed to align over exon-borders since it then would be unspecific towards unprocessed RNA with introns. Especially in genotyping, it can be a good idea to pre-amplify your template in a PCR reaction to get rid of surrounding mRNA that the primer/probes could bind unspecifically. This is not a great concern when amplifying mRNA since you have designed your primers over exon-junctions and you should have done a sequence alignment test of your primer/probes for unspecific binding.

Real-Time qPCR can generate much valuable data; you can run many specific markers for your area of interest and manipulate cell cultures to investigate interactions of stimuli and blockers. It is however important to remember that not all mRNA makes it to the secreted or expressed protein, downhill reactions do still occur.

Statistics

A number of statistical methods have been used in the projects of this thesis. Common for most of them is that we have been using nonparametric tests. We did not observe Gaussian

distribution of our sample variables even after logarithmic distribution, thus chose nonparametric tests. Parametric tests can be used even in small samples if the sample is chosen from a greater population in which Gaussian distribution is expected. As it is hard to determine if the population is of Gaussian distribution from small subsamples, we choose not to make such an assumption and use non-parametric test.

Two groups were compared by Mann-Whitney *U* test and three or more groups were compared with Kruskal-Wallis test for unpaired observations. Spearman's rank correlation test was used when relating two variables to each other non-parametrically. Chi-square test was used for categorical variables when no category had an expected count less than five, and the Fisher's exact test has applied when at least one category had an expected count less than five. Paired observations of two groups were calculated by Wilcoxon Signed Ranks test. A probability level of $p<0.05$ was considered as statistically significant whereas $p<0.1$ was regarded as tendencies.

Comparison of multiple genotype variables and T1D progression in Paper III was done by employing a 7-way analysis of variance. A probability level of $p<0.05$ was considered to be statistically significant, whereas p-value of $p<0.1$ was regarded as a tendency. In the analysis of variance, spontaneous and stimulated cytokine responses were used as response variables and gene polymorphisms and time were used as explaining variables. A model for post hoc adjustments was employed to reduce the risk of mass significance errors. Results were accepted as significant only if at least three explaining variables (polymorphisms) showed p-value<0.05 or for response variables (cytokines) at least two.

Calculations were performed in Statview 5.0.1 for Macintosh (Abacus Concepts Inc. Berkeley CA, USA), SPSS for Windows v14 (SPSS Inc. Chicago, IL, USA), GraphPad for Windows v4.03 (GraphPad Software Inc. San Diego, CA USA) and in Minitab Inc. PA, USA).

Results & Discussion

Paper I and III

In these two papers we studied HLA Class II and CTLA-4 genetic influence on immune responses towards, T1D-associated autoantigens. There is nearly an abundance of studies where associations between genetic polymorphisms and T1D incidence are investigated. Not saying that those studies are not important. We were interested in the gap of knowledge of what actual effect these T1D polymorphisms have on the immune system in healthy children and T1D patients. Genetically predisposed children give us a hint of what might be going on prior to disease. By measuring antigen-specific immune responses in healthy and T1D children we aimed to exploring interactions between immunological responses and genetic risk in two populations representing healthy children to four year after onset of T1D.

Descriptive

Two populations were used in these papers. Thirty-one T1D patients were recruited at the pediatric clinic at the Linköping University Hospital. The children were between 3-17 years old (average 10 years) at the time of sampling and, 16 were male and 14 female. Samples were collected at 0-3 months (sample (S)1), 10-18 months (S2) and 20-48 months (S3) after diagnosis. At medium duration (S2) only sample from 20 individuals were obtained.

The healthy control population was 7-15 years old and 32 male and 26 female. The studied individuals were recruited from a public school in the county of Östergötland. After individual and parents' consents blood samples were taken with a maximum of three boys and three girls in each school class. The blood samples were taken during a restricted time period and during morning hours to avoid time of day influences. The children were asked to fill out a form together with their parents regarding their own and the family's health. Children with an ongoing infection, atopy, celiac disease, T1D or autoimmune diseases or in close family were excluded from the study.

ELISPOT results

Th1 and Th2-like cytokine responses were measured as the median number of PBMCs secreting IFN-γ and IL-4 respectively, and in some cases also as the IFN-γ/IL-4 ratio. This latter might seem redundant since the data already are available in the other graphs. However since it is believed that Th1 and Th2 can cross regulate and the ratio between these cytokine seem to have a biological relevance, we chose to offer this clarification to the reader in some cases. Spontaneous secretion was subtracted from antigen-stimulated secretion in order to obtain specific response and normalise against "background noise".

A panel of T1D-associated antigens consisting of GAD_{65}, GAD_{65}-peptide a.a. 247-279, IA-2 and HSP60-peptide (DiaPep277) were used to stimulate the samples. All samples where positive spots were expressed in negative wells (no cells, only medium) were excluded. Samples in which positive (PHA stimulation) wells showed low response, were also excluded. In most cases, a second aliquot of the same sample could be used to replace the sample lost. Generally, all antigens induced IFN-γ response of similar magnitude whereas spontaneous IL-4 secretion was low and remained so also after antigen stimulations.

CTLA-4 +49A/G

Healthy children showed similar IFN-γ responses towards the selected antigens in the different CTLA-4 +49A/G genotype groups except when stimulated with HSP60-peptide. Risk GG-allele carriers showed significant lower responses towards this peptide compared to mixed AG (p=0.04) genotype and protective AA (p=0.02) (Fig 10). This particular peptide of HSP (DiaPep277) is used in a T1D intervention trial [128] where a protective Th2 phenotype is observed in HSP-peptide reactive T-cells after treatment. In our results we find a risk-associated Th1 phenotype response to HSP-peptide in CTLA-4 +49GG-allele individuals, well in line results from DiaPep277 trial. Comparing these results to T1D patients we observe that all healthy allele groups clearly distinguish themselves from T1D patients (Fig 8). Median response in patients was very close to zero, suggesting that these individuals are close to unresponsive to this peptide. IL-4 responses to the same peptide were of the same quantities in all allele groups of healthy children and in T1D it was very close to zero (data not shown). Intracellular HSP-60 peptide are present in cells that are involved in T1D

pathogenesis, but reactivity of the protein could be a sign of pathogenesis as well as a consequence of it. Analysis of variance also identified HSP60-peptide as a sensitive biomarker for T1D risk genes (data not shown).

Healthy children with CTLA-4 +49 AG ($p<0.02$) and protective AA alleles ($p=0.01$) distinguished themselves in GAD_{65}-induced IFN-γ responses at all studied time points when compared to T1D patients (Fig 9).

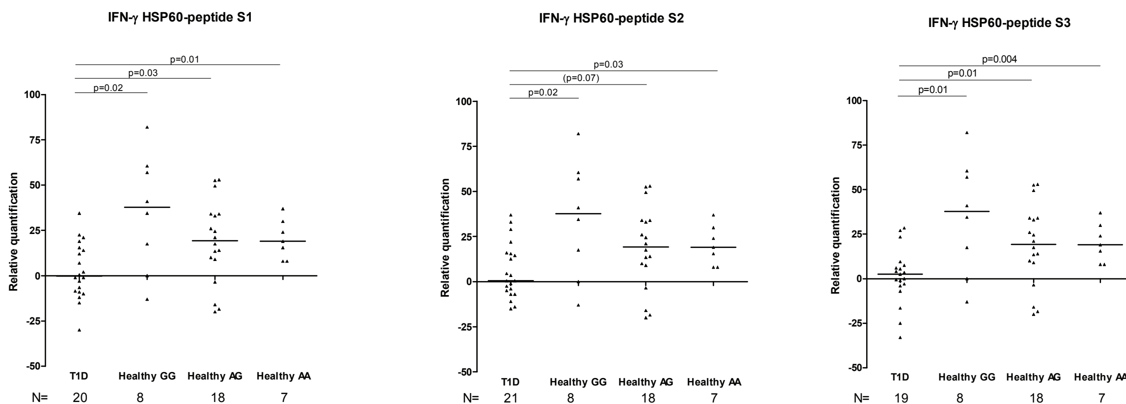

Figure 8. HSP60-peptide induced IFN-γ responses in CTLA-4 +49A/G subgroups

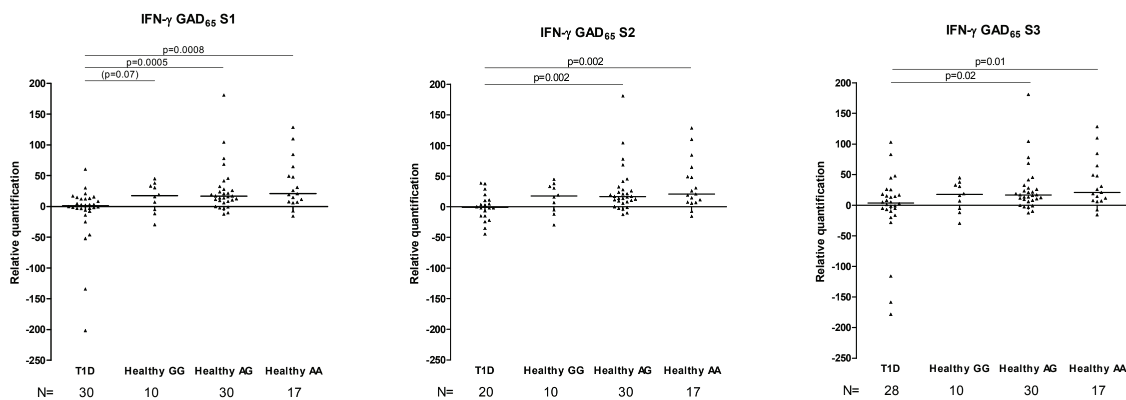

Figure 9. GAD_{65} induced IFN-γ responses in CTLA-4 +49A/G subgroups

IL-4 GAD_{65} responses in healthy individuals showed to be less pronounced in risk GG allele carriers than in protective AA ($p=0.02$). This interaction could be interpreted as a loss of protective Th2 phenotype response to one of the main T1D pathogens. However, GAD_{65} responses are known to vary during different phases of T1D development and in different HLA Class II risk groups. It might be too early to draw conclusions from this single observation although it seems important and well worth further investigation.

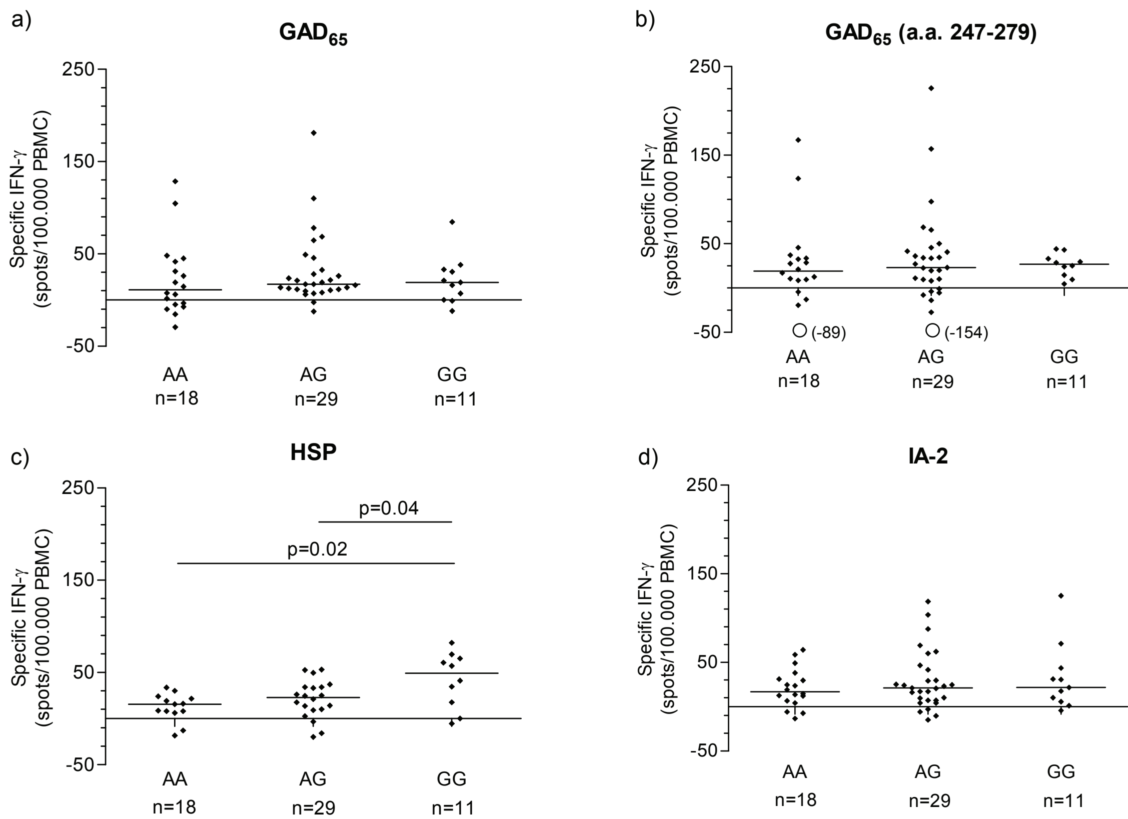

Fig 10. Antigen induced IFN-γ response in CTLA-4+49A/G subgroups

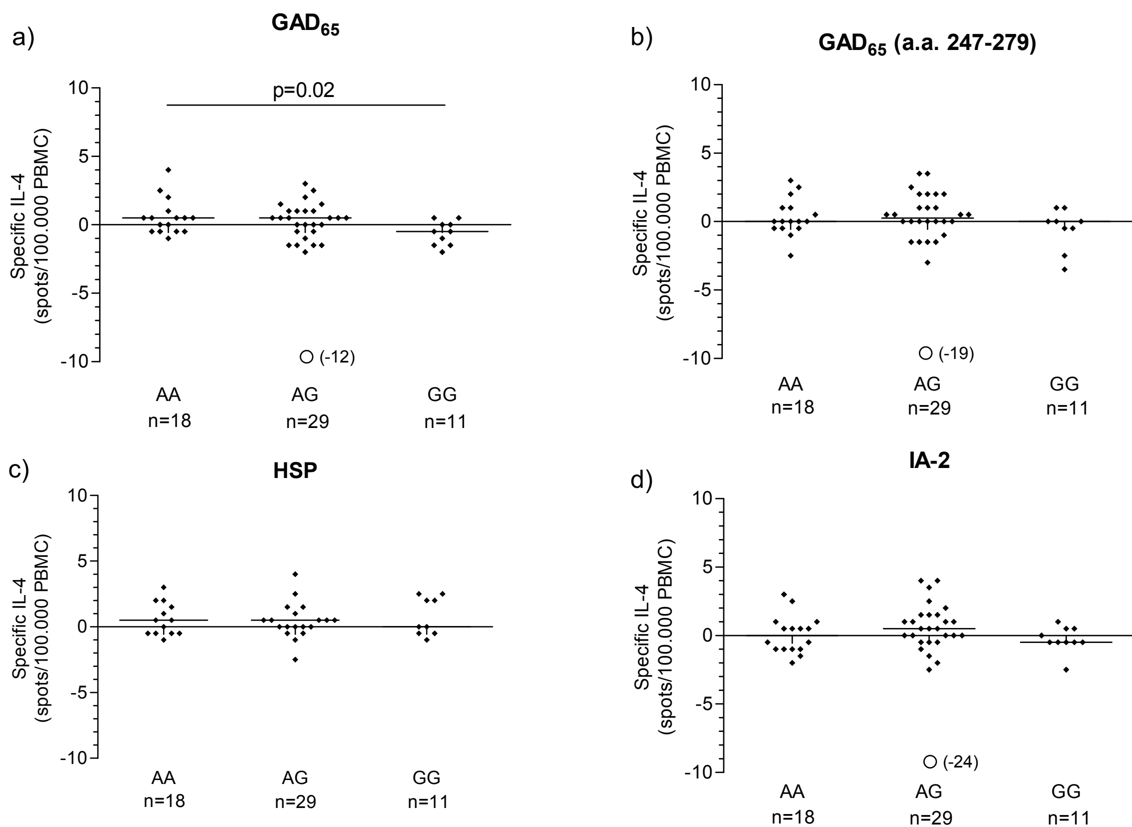

Figure 11. Antigen induced IL-4 response in CTLA-4 +49A/G subgroups

HLA Class II

Spontaneous IFN-γ/IL-4 ratio showed a Th1-dominance in risk ($p=0.01$) and neutral HLA class II individuals ($p=0.03$) compared to risk individuals with protective DQ6 allele (Fig 12). DQ6 is protective in T1D but at the same time a risk allele for MS [96]. Spontaneous secretion is hard to attribute to a certain process. Although MS-predisposing immunological effects are not expected to be present so early in life there could be a DQ6-related effect on Th1/Th2 balance. DQ6 protection of T1D is not completely understood and it could of course also be that the T1D “brake” is stronger than neutral resistance to Th1 dominance. This observation is matched by the increased IL-4 response towards IA-2 stimulation where HLA protective allele carriers also differed from risk allele ($p=0.05$), where neutral carriers did not.

IFN-γ GAD_{65}-responses was shown to be higher in healthy children neutral to T1D HLA Class II risk compared to risk individuals ($p=0.01$) (Fig 13). The increased IFN-γ GAD_{65}-response seems to be a protective characteristic, since we also observed that risk and protective risk children have a higher response than T1D patients ($p=0.0001$ and $p=0.01$ respectively) (Fig 13). This pattern was similar except that DR4-positive individuals showed higher IFN-γ responses than T1D patients. The response-protective hypothesis is supported by previous findings by our group [129-132]. It might be argued that even a Th1-response might be protective since it could be a sign of immune response towards, GAD_{65}. The speculative protection of IFN-γ response is supported by findings that IFN-γ producing cells are less frequent at T1D onset [133] and in an animal model IFN-γ responding cells to GAD_{65}-peptide (286-300) was found to be T1D protective [134]. The specific response could be associated with a regulatory response in an individual with active peripheral tolerance. In contrast, a loss of Th1-response might reflect a parallel capitulation of regulatory processes towards a central T1D antigen. In the case of T1D, it could of course also reflect that there are no antigen-containing cells left for Th1-asssociated autoimmune attack. It should however be noted that these are just speculations, since a similar set of arguments could have been used to explain an opposite finding. There are also questions about Th17-associated activity in this setting that remain to be investigated. Although statistics not allow us to draw final conclusion about the similarities between healthy individual both CTLA-4 +49A/G and HLA risk Th1-responses and T1D patients, it is tempting to speculate that these observations could reflect the pathogenesis. Speculatively it could be possible that HLA risk alleles predispose an individual

for T1D by lowering GAD_{65} responsiveness, and thereby open for a triggering event that starts the autoimmune process.

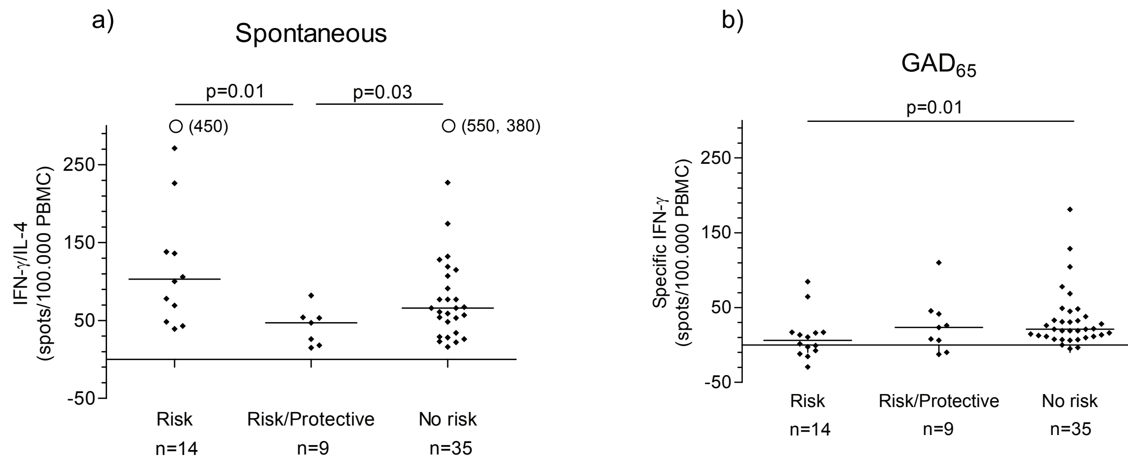

Fig 12. Spontaneous IFNγ/IL-4 ratio and GAD_{65} induced IFN-γ response in HLA risk subgroups

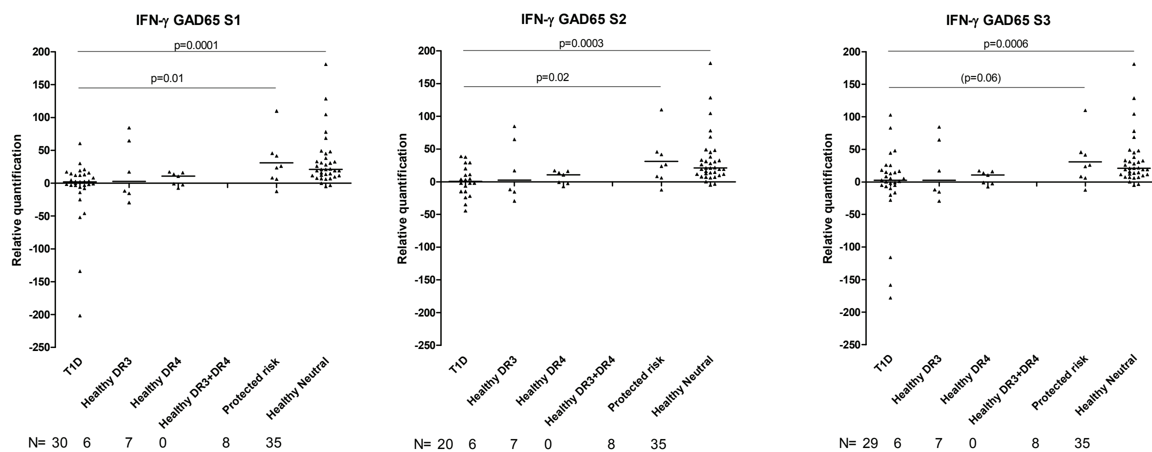

Fig 13. GAD_{65} induced IFN-γ responses in HLA Risk subgroups

HLA Class II and CTLA-4 were not correlated, as observed in previous studies [107]. Although both polymorphisms increase risk of T1D they are likely to be part of different pathways of pathogenesis. HLA is the major T1D genetic risk factor although it is interesting that we can observe a CTLA-4 +49A/G-associated affect on the central T1D antigen-responses. In summary, we have observations that indicate a genetic risk-associated reduced immune response that could be part of T1D pathogenesis.

Paper II

In an additional attempt of investigating CTLA-4 and HLA Class II effects on the immune system we made a study where we targeted Treg cells. As an impaired peripheral tolerance has been shown to autoimmunity and T1D in mice we wanted to explore in what manner HLA and CTLA-4 could affect CTLA-4 expression in Th cell and Treg population. We chose to recruit healthy five year old children to investigate markers of T1D associated with peripheral tolerance.

Descriptive

Fresh blood samples from the ABIS study were used in this project. Samples from 68 5-year-old children were selected randomly during the period of July 2003 to February 2004. As an initial pilot-test showed that samples older that 24 hours had bad quality those samples were excluded from the study. Although the T1D/MS effect of DQ6 on two different autoimmune diseases suggest that the allele determines disease-specific factor of the adaptive immune response, antigen-specific response is less likely associated with more general phenomenon such as the T-reg phenotype. However, we considered the interpretation of the data in relation to DQ6 complicated due in regard of autoimmunity. Thus to isolate the effect of HLA we decided to exclude the individuals with DQ6 allele resulting in a population of 47 healthy children.

The children did not have T1D or autoantibodies at the time the samples were taken and at the update of the ABIS database summer 2005 and none of the children included had diagnosed been diagnosed with T1D.

Gating strategy

Lymphocyte population gate was selected from the PBMC population based on size (Forward scatter) and granularity (Side scatter) as well as the location of the CD3 positive population (Fig 14). The lymphocyte population was then plotted for CD4 and the lymphocyte marker of interest. $CD4^{+}CD25^{high}$-gate was selected as the higher intensity $CD4^{+}CD25^{+}$-population (Fig 15). This latter method of defining the $CD25^{high}$-population has been subject to debate. Now commercially available antibodies against FOXP3 can further assist the selection of the Treg cells. However, at the time we could not use these and thus decided to use this somewhat

arbitrary method of definition. However, we had a run-in period of analyse to minimize subjective variability. As a reference we used the CD4-negative population, and sat the $CD25^{high}$-cut-off where that population did only have sparse expression of CD25 left. Another method is to use a fixed level of cut-off as a reference. We decided not to use this strategy since even small variances of where the CD^{25high}-population cut-off is set, can have a big impact on final outcome.

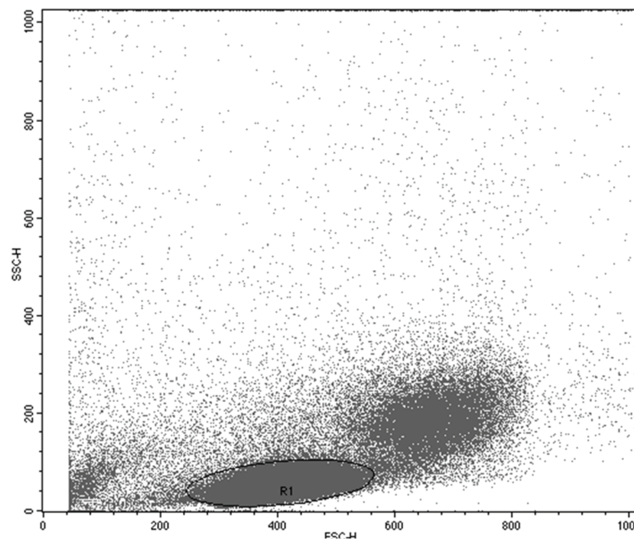

Fig 14. Lymphocyte gate was selected by size, granularity and the location of CD3+ cells

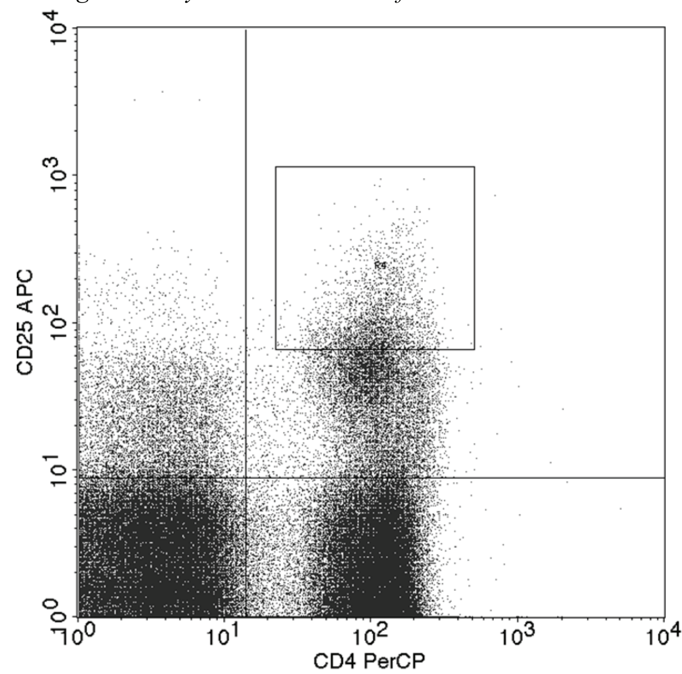

Fig 15. $CD25^{high}$ gate was selected where $CD4^-$ where getting sparse

T1D neutral HLA allele carriers showed to have higher percentages of CD4+ cells expressing intracellular CTLA-4 than both DQA1*05-DQB1*0201 (p=0.002) and DQB1*0302-positive children (p=0.04) (Fig 16). Parallel increased percentages of intracellular CTLA-4 were found in CD25^{high} population of cells in children with neutral genotype compared to children with DQA1*05-DQB1*0201 (p=0.002) and DQB1*0302-positive children (p=0.02) (Fig 17). Bearing in mind that 95% of children with T1D originate from this HLA risk background it is intriguing to speculate that they also have a reduced intracellular storage pool of CTLA-4 in both their general Th cell population and Treg cell population. Since CTLA-4 is involved in various processes of peripheral tolerance these results indicate that already from early age these healthy predisposed children have a reduced capacity to mount regulatory immune signals. Although differences in percentages are low, this could very well be of biological relevance.

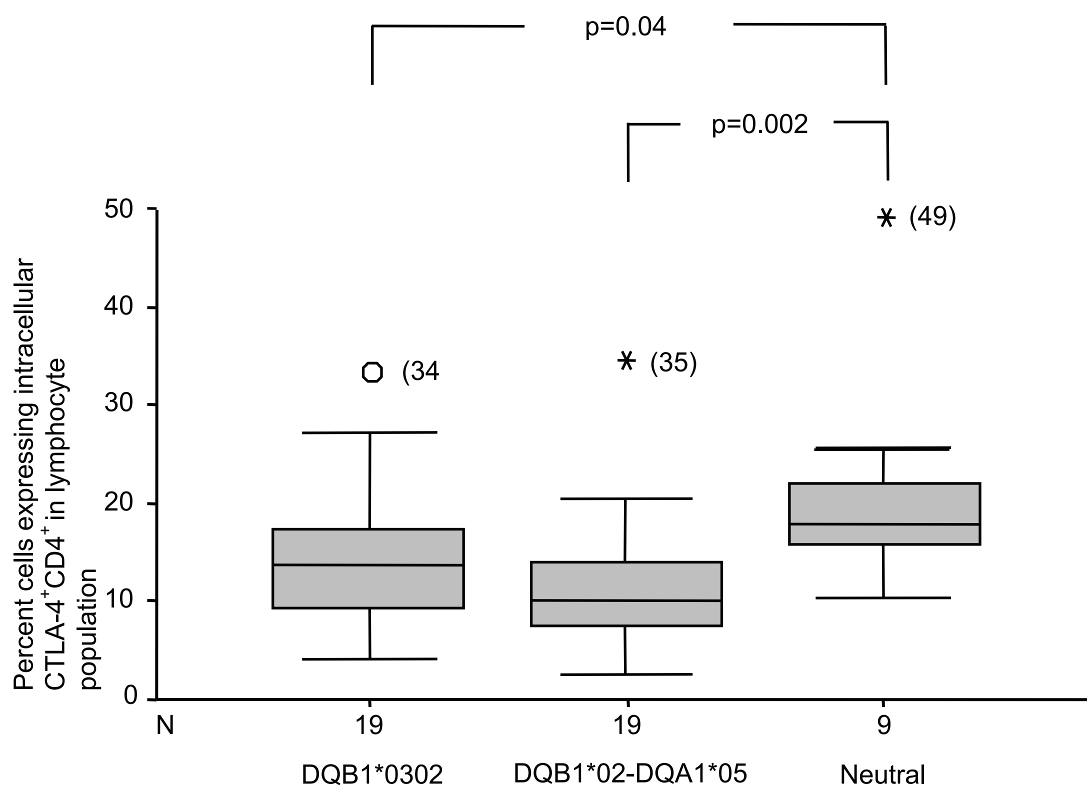

Fig 16. Percentage of cells expressing specific marker in HLA risk subgroups

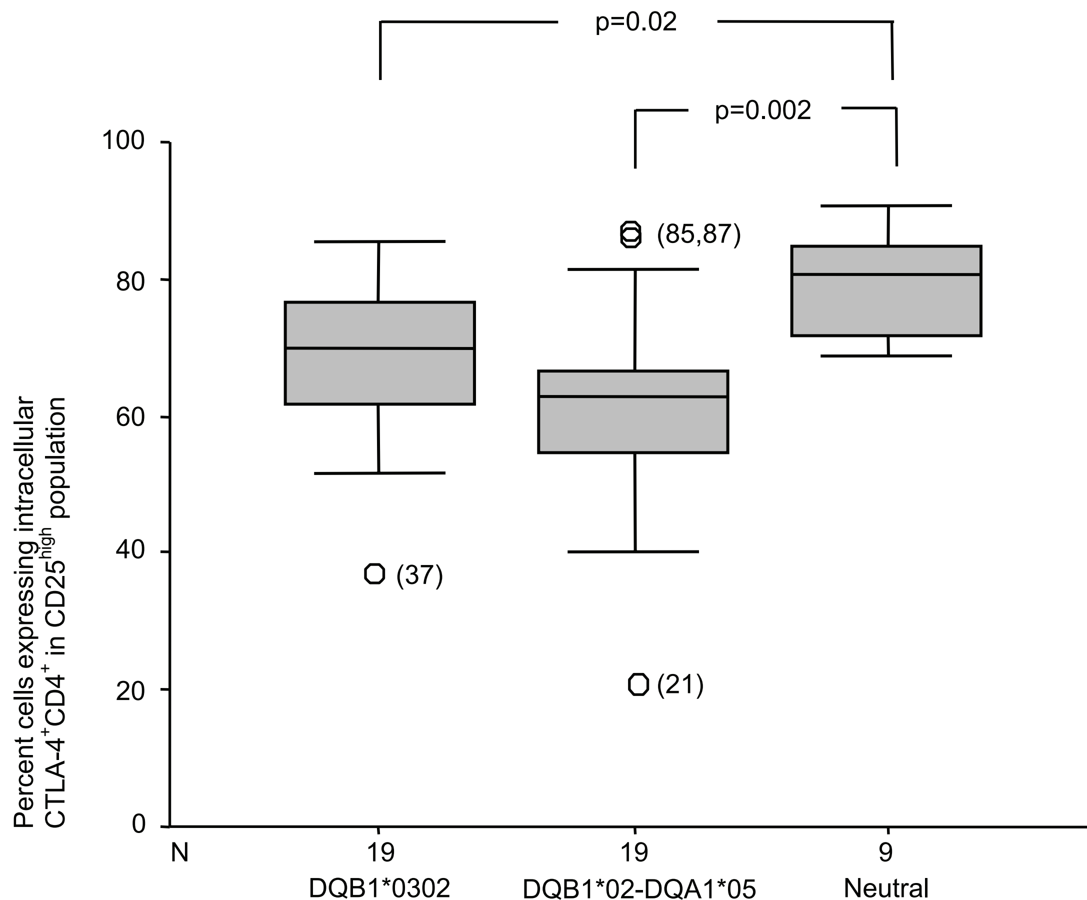

Fig 17. Percentage of cells expressing specific marker in HLA risk subgroups

Children with risk-associated CTLA-4 +49GG allele had significantly lower percentages of CD4 cells expressing intracellular CTLA-4 in the lymphocyte population compared to AA allele carriers (p=0.03) (Fig 18). This tended to be consistent in the $CD25^{high}$ population where GG carriers had lower intracellular pools of CTLA-4 than AA (p=0.06) (Fig 19). As in HLA risk, this relationship might reflect a reduced capacity to mount regulatory CTLA-4 responses in CTLA-4 +49A/G risk individuals. It could be argued that the HLA-associated decrease in intracellular CTLA-4 affect what we observe when we compare CTLA-4 polymorphism. This is of course a concern, but we do observe this effect in a population where T1D neutral genotype is also included. This is also the biological setting, we know that both polymorphisms are associated with T1D autoimmunity, and even though these variables might not act along the same pathway, the effect of risk are certain to be additive. CTLA-4 +49A/G polymorphism has also been shown to be associated with other autoimmune diseases so there are no reasons to disregard this polymorphism effect observed in this setting.

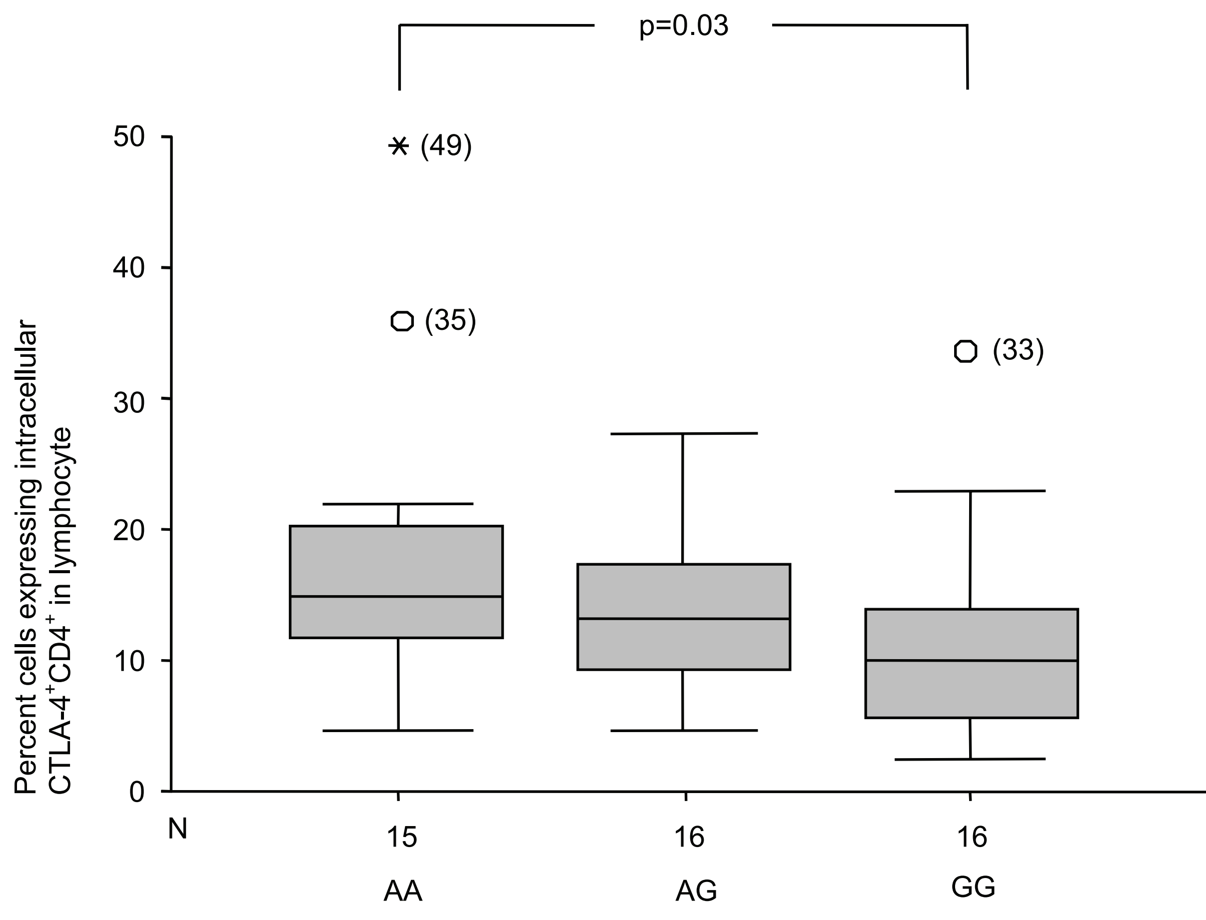

Fig 18. Percentage of cells expressing specific marker in CTLA-4 +49A/Gsubgroups

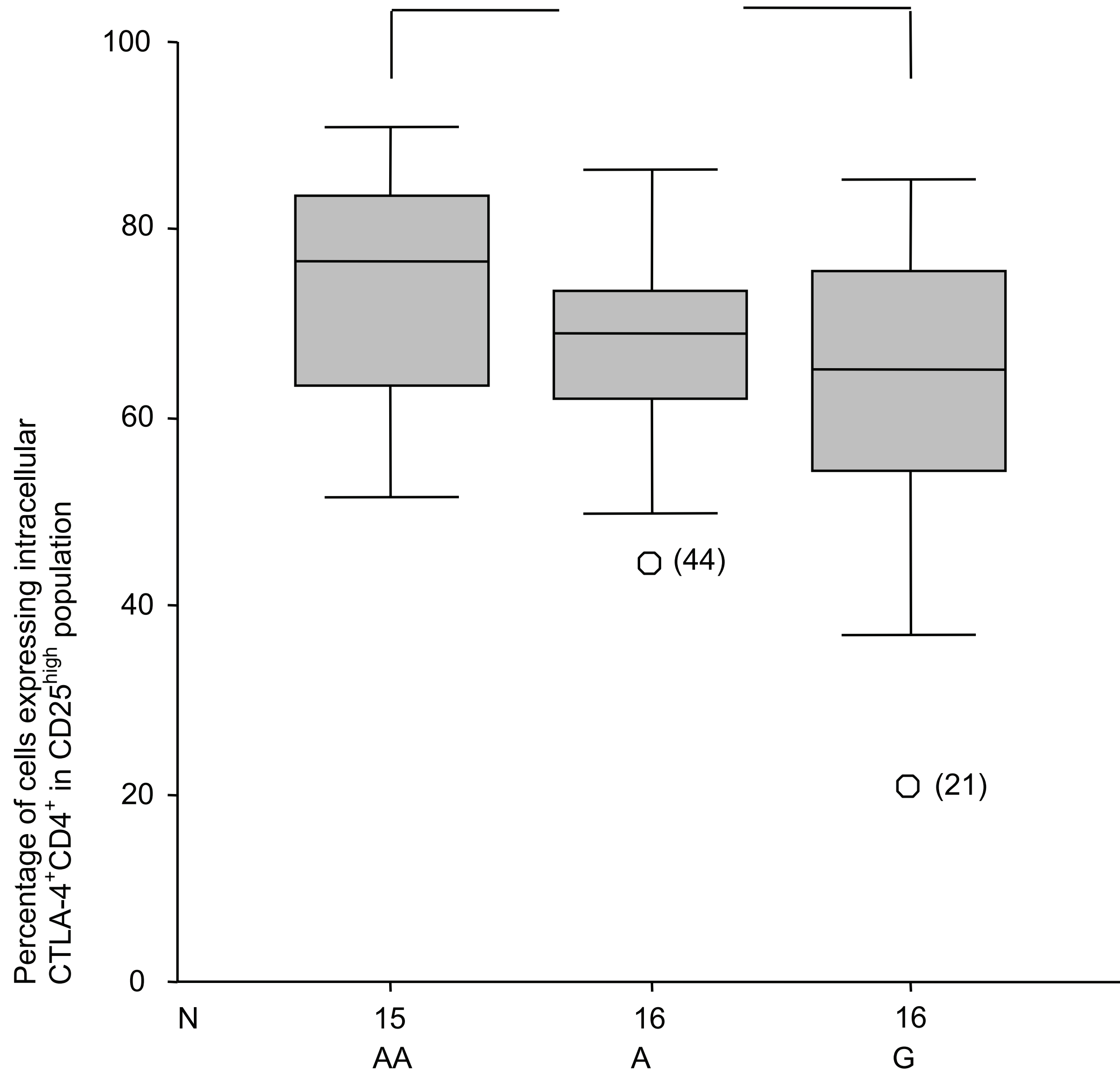

Fig 19. Percentage of cells expressing specific marker in CTLA-4 +49A/Gsubgroups

A small subset of samples (n=16) was selected based on availability of material for Real Time PCR of mRNA. The results showed that the percentages of $CD25^{high}$ population correlated negatively with CTLA-4 mRNA expression (r=-0.56, p=0.03) (Fig 20). The results are quite unexpected. Even though Treg constitutively express CTLA-4 the recorded changes (as a result of different percentages of Treg) of mRNA are influenced by many other cells in the PBMC population. Our theory is that an increase in percentage of Treg cells corresponds to a "healthier/normal" immune system. In this normal state, the Treg cells are anergic, and influence other PBMC cells to be that as well. Since mRNA expression is up regulated upon stimulation it seems these low levels are corresponding to the anergic cells that have no need

to combat autoimmune processes. The individuals with lower percentages of Treg cells might instead have a problem controlling autoreactive cells, resulting in an activation of T-cells, and thus, an increase in CTLA-4 mRNA transcription. We can not know in this methodological design if we measure T-cells or induced regulatory T-cells.

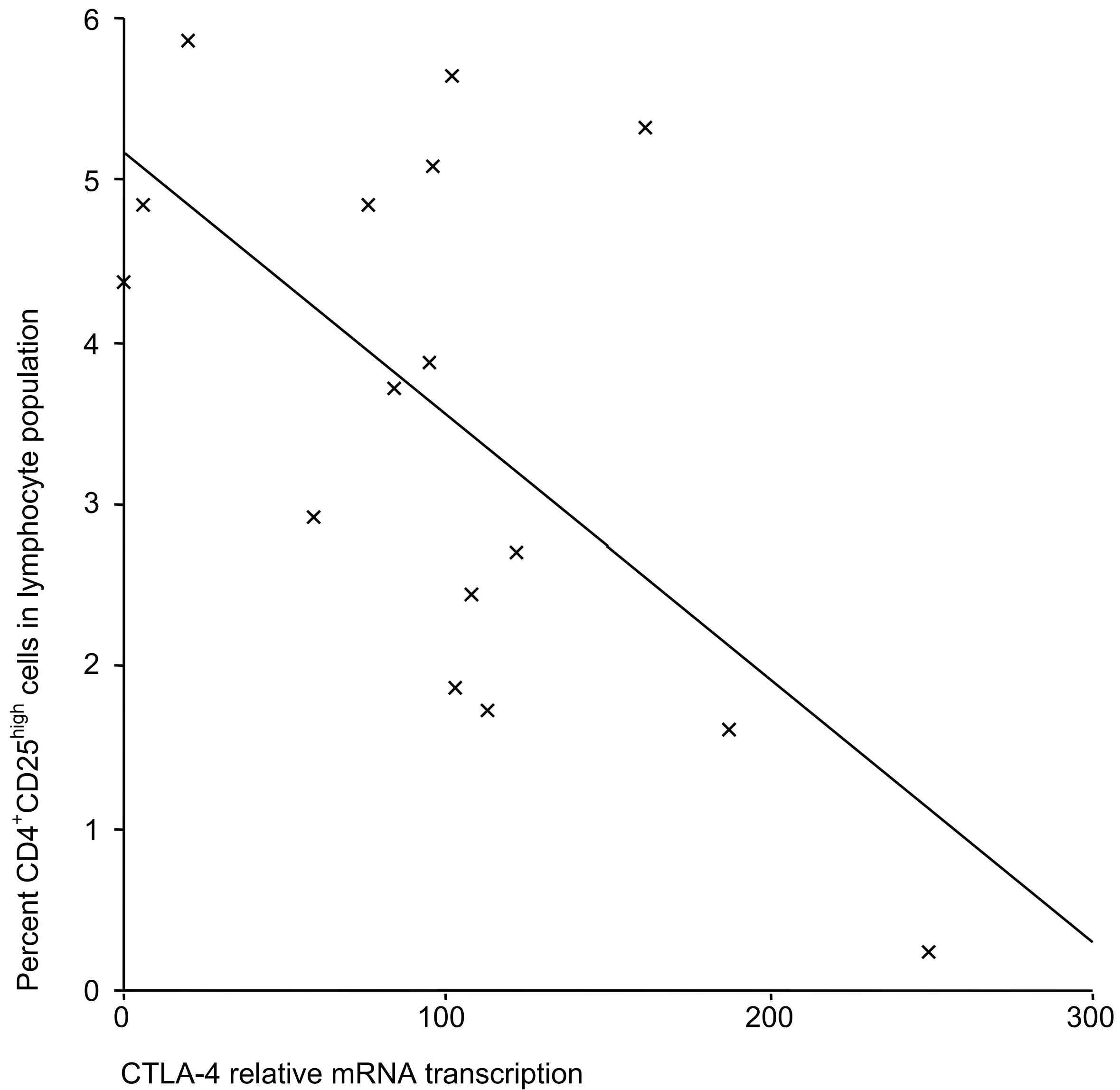

Fig 20. Percentage of lymphocytes negatively correlated againt CTLA-4 mRNA transcription in PBMC population

Although the sample size was too small, we did a Chi^2-test for correlations between HLA and CTLA-4 genotypes but found no significant results. As previously argued this seems plausible since CTLA-4 and HLA genes are situated on different chromosomes and most likely have independent pathways of predisposing for T1D.

The aim at flow cytometry analysis was to collect as many cells as possible, using an acquisition cut-off at 100 000 PBMCs, but usually the sample of blood did not give such yield. The sample volume available for this study varied. This resulted in a sample yield between 10.000 and 100.000 cells per flow cytometry test tube (one antibody combination). An absolute number of Treg cells would vary even though the relative number of Treg cells was the same. We could have employed this design if we would have set the acquisition to a lower number of cells, like 20.000, but then much information would have been lost. The percentage of CTLA-4 expressing cells in relation to $CD4^+$ or $CD4^+CD25^{high}$ population is relevant since we study the "functional" population of relative cells.

We did not find any CTLA-4 +49A/G effect on CTLA-4 mRNA. We speculate that CLTA-4 mRNA is not necessarily correlated to percentage of cells positive for the protein. The polymorphism could be affecting the development of Treg in thymus or during peripheral differentiation. CTLA-4 mRNA is regulated very quickly in the cells whereas the intracellular CTLA-4 storage pool is unlikely to follow the same kinetics. It should also be noted that the mRNA CTLA-4 has been measured in PBMC, whereas CTLA-4 protein expression is measured in $CD4^+$ cells. CTLA-4 is mostly expressed in $CD4^+$ cells but has also been found in CD8+ and B-cells.

In summary we observe a reduced percentage of cells expressing intracellular CTLA-4 in the lymphocyte and the Treg population in individuals with genetic predisposition to T1D. We conclude that these individuals might have reduced capacity to mount regulatory responses, which could be part of early T1D pathogenesis.

Paper IV

Extracorporeal photopheresis (ECP) is a therapeutic method for treatment of autoimmune disorders. In previous studies performed at the University Hospital of Linköping, ECP treatment had shown positive immunological effects in children with recent onset T1D. In this paper we wanted to further investigate markers associated with peripheral regulation and their role in T1D ECP intervention.

Descriptive

Frozen cell and serum samples from the original ECP trail was available. Twenty children with recent onset T1D had been selected and randomly allocated to placebo or active treatment group. Ten recent onset T1D children (ages 10-17 years) received active treatment, and ten children (matched by gender and age) received placebo treatment. The two groups showed no differences at sample zero in mRNA expression of FOXP3, CTLA-4, sCTLA-4 and TGF-β, with one exception. PHA-stimulated FOXP3 expression tended to be higher in photopheresis than in the placebo control group (p=0.06). Spontaneous CTLA-4 mRNA expression increased during the course of the study in both the active (p=0.04) and placebo treated group (p=0.02) between day 0 and day 90. Samples were stimulated with a GAD_{65}-peptide a.a. 247-279 and the mitogen PHA. Spontaneous FOXP3 and TGF-β mRNA expression correlated in both groups, both prior (r=0.66, p=0.002) and after ECP treatment (r=0.7, p=0.001).

Expression of Treg associated markers after treatment

ECP treated children showed limited changes for the studied Treg associated markers. No changes were detected in relative GAD_{65}-peptide or PHA-induced CTLA-4 transcription. Spontaneous, GAD_{65}-peptide and PHA-induced sCTLA-4 transcription also seemed to be stable during the treatment period. TGF-β mRNA showed no detectable transcription differences in spontaneous, GAD_{65}-peptide or PHA stimulated relative transcription (Fig 21).

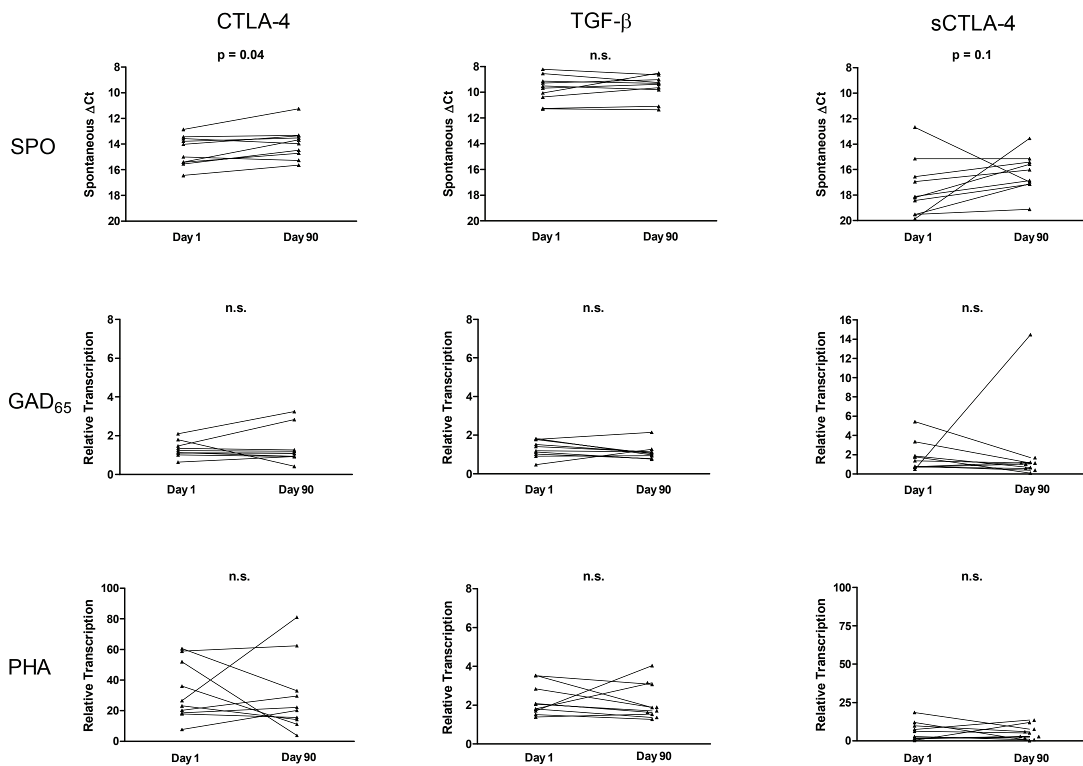

Figure 21. Spontaneous and stimulated mRNA transcription of Treg markers in ECP treated children

Placebo controls showed a completely different course of development at the same period of time. Relative transcription of the GAD_{65}-peptide – and PHA induced CTLA-4 mRNA was decreased in the placebo group after 90 days (p=0.02 and p=0.03, respectively) (Fig 22). In parallel, spontaneous sCTLA-4 mRNA expression tended to increase in the placebo treated group (p=0.08) (Fig 22).

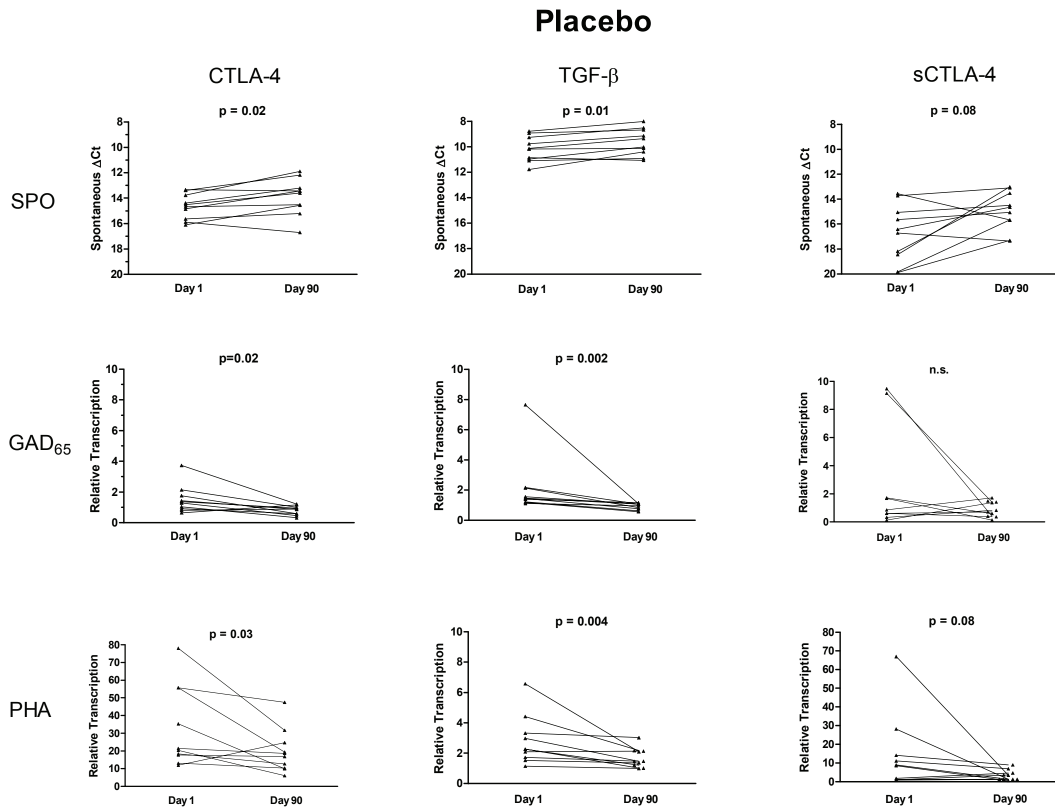

Figure 22. Spontaneous and stimulated mRNA transcription of Treg markers in placebo treated children

Paired test of transcription in the two groups indicates that Treg associated transcription-markers decreases in individuals receiving placebo treatment. To further justify our conclusions we tested if the relative changes in each group were greater in the placebo group that ECP treated group. We observed that relative changes were significantly greater in GAD_{65}-peptide stimulated CTLA-4 and TGF-β mRNA placebo control group than in ECP treated group (Fig 23, 24).

Bear in mind that the placebo treated individuals follows the course of T1D pathogenesis that is expected during the first three months after diagnosis. At diagnosis it is possible that there are some residual insulin producing beta cells in the pancreas but these cells are expected to be destroyed by autoimmune reactions. We detect an association of disease-specific GAD_{65}-

peptide responses of CTLA-4 mRNA. This observation is followed by a parallel reduction of TGF-β responses towards the same T1D antigen. These parameters were counteracted by the photopheresis treatment since we fail to detect any such reduced response in that group.

We interpret our collected results to support the thesis that ECP induces CTLA-4 associated tolerance. Together with TGF-β an immune-regulating setting is maintained. This is specific against GAD_{65}-peptide, a central T1D antigen. Of general interest we also find a correlation of FOXP3 and TGF-β, well in line with current Treg biology understanding. We conclude that ECP acts to maintain immune regulation and thereby can inhibit spontaneous islet autoimmunity. Although clinical outcome of ECP treatment were certainly limited, ECP might serve as an effect booster for other immune intervention candidate therapies.

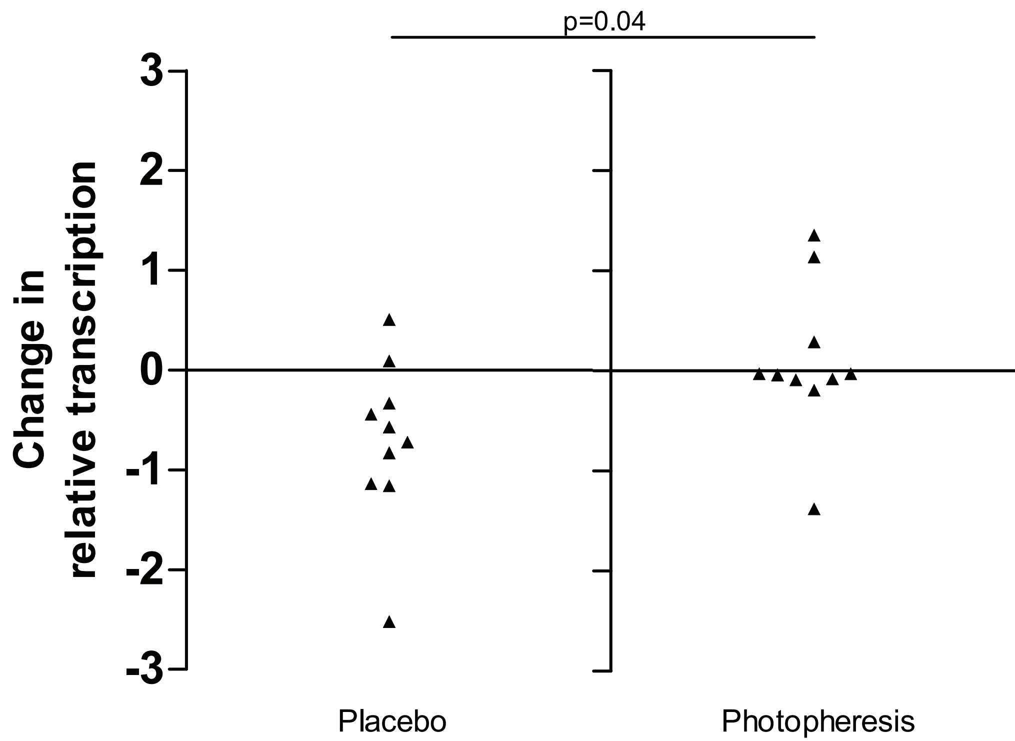

Fig 23. Comparison of relative pre treatment to post treatment transcription changes in the Placebo and ECP treated group.

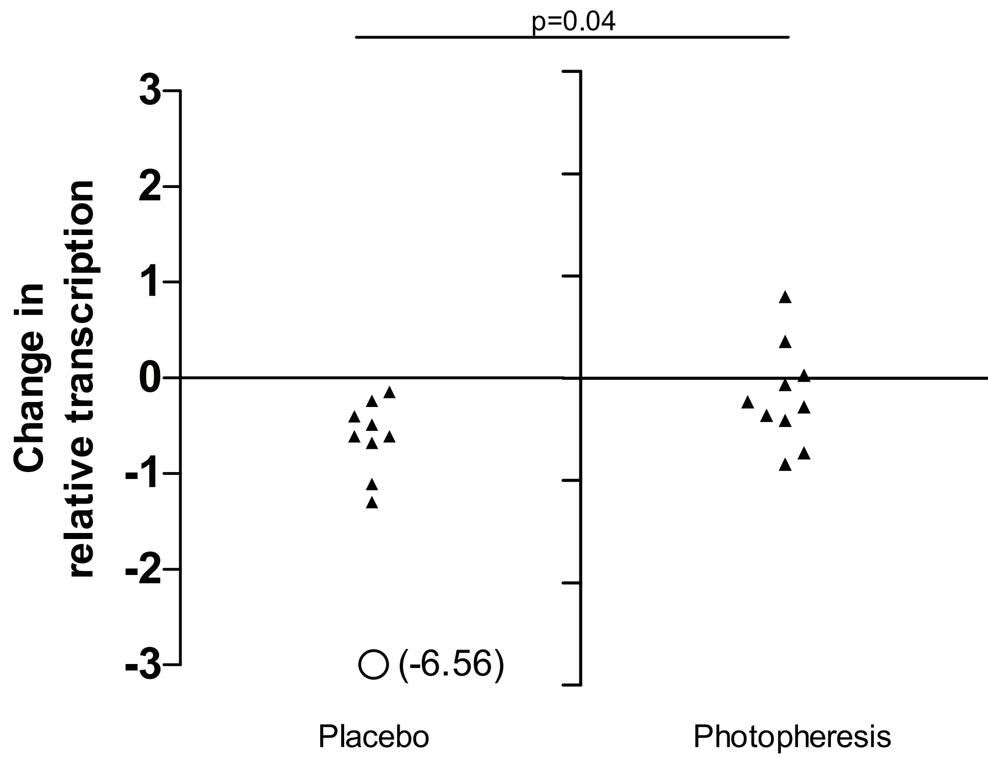

Fig 23. Comparison of relative pre treatment to post treatment transcription changes in the Placebo and ECP treated group.

Summary and Conclusion

T1D is a complex and multifactorial disease. In this field of research, how do you distinguish one predisposing variable's effect from another? One method of course is to isolate your variables of interest in a model where you control the environment. This can be done in a cell line *in vitro* setting or in an animal model. The benefits are that you can be quite certain what the cause of your outcome is, but in the end, do you know what relevance it holds in a clinical setting and in humans?

In the other end you can do large population investigations, looking for environmental factors associated with the disease, or if you combine it with genetics, genes associated with disease. Then, would you know how this factor that you have found predisposes for disease?

It is in the research area in between these we have made an effort to investigate immune responses central for T1D development in healthy individuals and T1D patients. In the healthy population we have been focusing on two genes important in T1D, HLA Class II and CTLA-4.

The results indicate that there are immune response differences in T1D patients and healthy children might not be so surprising. But in the case of HLA Class II risk in healthy children, this is the group where T1D patients are "recruited". If 95% of all T1D cases are either DR3 or DR4 positive and there is a possibility that there are yet unmapped risk-alleles in this region, could we not say that HLA Class II risk gene can been seen as a requirement for the development of T1D?

Paper I, II and III address this question. In paper II we see that in the population of T1D patients are recruited, there is a general reduction in CTLA-4 storage pools in both the lymphocyte and Treg population in healthy children. It would be easy to speculate that this reduction in the capacity to mount tolerance induction by CTLA-4 could be setting the stage for autoimmunity. Similarly our results from paper III indicate that HLA risk lowers immunological responses towards T1D antigen GAD_{65} to a phenotype that resembles the one of T1D patients. Speculatively this could also be part of the stage-setting for triggering events causing T1D.

Could it be that once the stage is set, one out of several triggers can cause T1D? Even more intriguing, could an alternative trigger, cause another autoimmune disease? Since T1D shares HLA risk genotypes with celiac disease [93, 95], maybe the nature and timing of the trigger could determine the resulting disease? Further speculating, could this explain the double nature of HLA DQ6? Could DQ6 set at protective stage for T1D development at young age but a predisposing for MS at older age?

Although not of the same magnitude as HLA Class II risk, we see a T1D-associated effect of CTLA-4 +49A/G polymorphism as well. GG risk allele individuals show smaller differences in GAD_{65} IFN-γ response compared to T1D patients, that mixed AG and protective GG allele carriers does. The intracellular storage pool of CTLA-4 protein is also lower in the lymphocyte as well as Treg population in healthy GG risk carriers, than protective AA carriers. Even if CTLA-4 +49A/G is a minor factor in T1D predisposition, we can measure effects that is possibly associated with T1D pathogenesis, and similar to the HLA risk alleles' effects.

In paper IV we evaluate if a clinical trail using ECP has any effect on T1D-specific immune regulation by monitoring markers associated with Treg. Although clinical outcome was a disappointment in this trail some immunological effects was observed. A suppressive effect was observed in the ECP group as compared to placebo where $CD4^+$ and $CD8^+$ cell activation increased [135]. In our paper we further investigate these results and find parallel associations. ECP treatment act to maintain TGF-β and CTLA-4 responses towards GAD_{65}-peptide stimulations, compared to placebo treated group that decline in responsiveness. Although the clinical outcome of the treatment is certainly limited, we observe potent immune regulatory effect that might used to boost other immune modulating treatments.

In our projects we have been studying GAD_{65} in many settings. It is quite safe to say that it is a T1D antigen of major importance. It still remains uncertain exactly which reactions and of which signal is T1D protective and when. This is something that most certainly will be well studied in the Diamyd trial [136]. GAD_{65} is involved in many complicated interactions, and now that the Th17 cells have been introduced as potential players, the stage is somewhat reset.

There are many models used to help us understand T1D pathogenesis. The above arguments are in line with the multi-hit hypothesis [137]. T1D and T2D have also been suggested to be

different manifestations of the same disease [140-141]. Of course there are similarities between T1D and T2D, inflammation is an active component of both diseases. It would not be surprising to find autoimmune reactions in the inflamed tissue of T2D, but is the disease autoimmune? Does T2D meet the criteria for an autoimmune disease? Can cells be adoptively transferred to a healthy animal and cause disease? Are there immune genes that are implicated in the disease? It seems that the theory is at least so far, a bit weak. For those opposing this theory there is of course a problematic observation in the finding of Latend Autoimmune Diabetes in the Adult (LADA) patients. Genetic mapping in a recent publication argues that T1D and T2D clearly follow different lines of gene activity [89]. But do we really need to force the two diseases into one frame? If anything, what would be good for research is if we started to discuss autoimmune disease as one disease. T1D and T2D share important similarities in the clinic, but when it comes to research T1D has much more in common with other autoimmune diseases. If we need one unifying disease, should we not talk about "Autoimmune disease"?

There are several interesting T1D intervention trails going on now in the world. Diamyd, anti-CD3 and DiaPep 277 are all potential candidates for halting T1D progression [138]. Diamyd is designed to induce tolerance by a "vaccination" of GAD_{65} and is hopefully starting a phase III trial quite shortly. Anti-CD3 therapy is though to work by affecting auto-aggressive effector lymphocytes and promoting an environment that favours Treg generation [47, 138, 139]. DiaPep277 is derived from HSP-60. It is not known how it works, but a Th1 to Th2-like cytokine shift has been observed in humans in the clinical trials [128]. Hopefully one of these therapy candidates will be successful. As proposed by Bresson and Herrath, the therapies could be combined [138]. Maybe ECP should also be considered in such a combination therapy.

T1D incidence in the world is increasing. Clinical trials are very important. However, if we are to know what to look for, we need basic research as well. Immunological studies of the therapeutical effects are impossible without knowing what the normal course of T1D pathogenesis is. Hopefully a therapy candidate will make it, but it might not be possible to get a high success rate in patients already diagnosed with T1D. In order to be able to identify prediabetic patients it is instrumental that we study high-risk individuals and search for early markers of disease.

T1D is an interesting field of research, and exciting times lies ahead. And as Private Taylor says in the movie “Platoon”, the war is in us. Hopefully peace is not to far away.

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