Studies of immunological risk factors in type 1 diabetes

Jenny Walldén

Division of Pediatrics and Diabetes Research Centre
Department of Clinical and Experimental Medicin
Faculty of Health Sciences, Linköping University
SE-581 85 Linköping, Sweden

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"The greatest virtue of man is perhaps curiosity"

Anatole France

To my family,

with all my love
ABSTRACT

Background: Type 1 diabetes (T1D) is a chronic, autoimmune disease caused by a T cell mediated destruction of β-cells in pancreas. The development of T1D is determined by a combination of genetic susceptibility genes and environmental factors involved in the pathogenesis of T1D. This thesis aimed to investigate diverse environmental and immunological risk factors associated with the development of T1D. This was accomplished by comparing autoantibody development, T cell responses and the function of CD4⁺CD25⁺ regulatory T cells between healthy children, children at risk of T1D and T1D patients.

Results: Induction of autoantibodies in as young children as one year old, was associated with previously identified environmental risk factors of T1D, such as maternal gastroenteritis during pregnancy and early introduction of cow’s milk. We did not see any general increase in the activity of peripheral blood T_H subtypes in children with HLA class II risk haplotypes associated with T1D, nor were HLA class II risk haplotypes associated with any aberrant cytokine production in response to antigenic stimulation of peripheral blood mononuclear cells. However children with a HLA class II protective haplotype showed an increased production of IFN-γ in response to enteroviral stimulation. CTLA-4 polymorphisms connected with a risk of autoimmune disease were associated with enhanced production of IFN-γ. Healthy children with β-cell autoantibodies had a lower expression level of GATA-3 compared to health children with HLA risk genotype or children without risk. Instead, children with manifest T1D showed lower expression levels of T-bet, IL-12Rβ₁ and IL-4Rα. Both T1D and healthy children showed the same expression of the regulatory markers Foxp3, CTLA-4 and ICOS in peripheral blood mononuclear cells, and the amount of CD4⁺CD25⁺ T cells did neither reveal any differences. The regulatory T cells seemed also to be functional in children with T1D, since increased proliferation after depletion of CD4⁺CD25⁺ T cells from PBMC was demonstrated in T1D as well as in healthy children. However, T1D children did have more intracellular CTLA-4 per CD4⁺CD25⁺ T cell, increased levels of serum C-reactive protein and higher spontaneous expression of IFN-γ in CD25depleted PBMC, all which are signs of activation of the immune system. This suggests a normal or enhanced functional activity of regulatory T cells in T1D at diagnosis.

Conclusions: Our findings emphasize that environmental risk factors do have a role in the development of β-cell autoimmunity. Our results do not support a systemic activation of the immune system in pre-diabetes or T1D, but instead a possible up-regulation of regulatory mechanisms seems to occur after diagnosis of T1D, which probably tries to dampen the autoimmune reaction taking place.
SAMMANFATTNING


**Resultat:** Produktionen av autoantikroppar kunde detekteras redan vid ett års ålder och relateras till olika miljöfaktorer i barnets omgivning. Till exempel finns det associationer mellan maginfluensa hos mamman under graviditet eller om barnet tidigt i livet får komjölksbaserad välling och utvecklingen av antikroppar hos barnet vid ett års ålder. Dessa autoantikroppar leder dock sällan till manifestation av diabetes.

Barn med genetisk risk för diabetes verkar inte ha någon generell obalans i det perifera immunsystemet och inte heller någon rubbad produktion av cytokiner efter stimulering av perifera mononucleära celler. Däremot verkar barn med en skyddande genetisk profil ha ett ökat IFN-γ svar mot enterovirus. CTLA-4 polymorfier associerade med ökad risk av autoimmuna sjukdomar gav en ökad produktion av IFN-γ.

En pågående autoimmun process mot de insulinproducerande cellerna i pankreas verkar påverka nivån av transkriptionsfaktorn GATA-3, då barn med autoantikroppar hade lägre nivåer av GATA-3. Barn med manifest diabetes hade istället en lägre nivå av IL-12Rβ1, IL-4RA och T-bet. Friska barn och barn med diabetes hade lika nivåer av de regulatoriska markörerna Foxp3, CTLA-4 och ICOS i perifera mononucleära celler, och även nivån av de regulatoriska CD4⁺CD25⁺ cellerna var lika. De regulatoriska T cellerna verkar dessutom vara funktionella, då cells från friska barn och diabetesbarn svarade på liknande sätt vid proliferationstester.

Vid insjuknandet av diabetes hade barnen ett aktiverat immunsystem, sett som högre nivåer av intracellulärt CTLA-4 per CD4⁺CD25³ T cell, högre nivåer av akutfasproteinet C reaktivt protein och ett högre uttryck av IFN-γ hos lymfocyter efter att CD25⁺ celler tagits bort.

**Slutsatser:** Våra fynd betonar vikten av miljöfaktorers påverkan av immunsystemet och utvecklingen av autoantikroppar. Vi kan däremot inte säga att det finns en systemisk aktivering av immun systemet innan sjukdomen framträder, men när väl diabetes utvecklats är immunsystemet aktiverat och denna aktivering verkar regulatoriska celler försöka dämpa.
ORIGINAL PUBLICATIONS

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

I. Wahlberg, J., Fredriksson J., Nikolic E., Vaarala O., Ludvigsson J. and the ABIS-study group
   Environmental factors related to the induction of beta-cell autoantibodies in 1-yr-old healthy children

II. Walldén J., Honkanen J., Ilonen J., Ludvigsson J. and Vaarala O.
    No evidence for activation of T_H1 of T_H17 pathways in unstimulated peripheral blood mononuclear cells from children with β-cell autoimmunity or T1D
    Journal of Inflammation Research, In press

III. Walldén J., Ilonen J., Roivainen M., Ludvigsson J., Vaarala O. and the ABIS-study group
    The effect of HLA genotypes or CTLA-4 polymorphism on cytokine response in healthy children
    Scandinavian Journal of Immunology 2008:68 (3):345-350

IV. Walldén J., Lundberg A., Ludvigsson J. and Vaarala O.
    Regulatory T-cells in children with type 1 diabetes
    Manuscript
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**ABBREVIATIONS**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABIS</td>
<td>all babies in south-east Sweden</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>cSMAC</td>
<td>central supra-molecular activation cluster</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte associates antigen 4</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CVB4</td>
<td>coxackie virus B4</td>
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<tr>
<td>DASP</td>
<td>diabetes autoantibody standardization program</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead/winged helix transcription factor</td>
</tr>
<tr>
<td>GAD$_{65}$(A)</td>
<td>glutamic acid decarboxylase (autoantibody)</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IA-2(A)</td>
<td>tyrosine phosphatase-like insulinoma antigen-2 (autoantibody)</td>
</tr>
<tr>
<td>IA-2ic</td>
<td>intracellular part of the IA-2 protein</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibody</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible co-stimulator</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-4R</td>
<td>interleukin-4 receptor</td>
</tr>
<tr>
<td>IL-12R</td>
<td>interleukin-12 receptor</td>
</tr>
<tr>
<td>iTreg</td>
<td>inducible regulatory T cell</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LYP</td>
<td>lymphoid tyrosine phosphatase</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescent intensity</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>nTreg</td>
<td>naturally regulatory T cell</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>pSMAC</td>
<td>peripheral supra-molecular activation cluster</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SPSS</td>
<td>statistical package for the social sciences</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activator of transcription</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>T-box</td>
<td>T-box expressed in T cells</td>
</tr>
<tr>
<td>Tc</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cells</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>regulatory T cells type 1</td>
</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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**REVIEW OF THE LITERATURE**

**Introduction to immunology**

*Basic immunology*

Immunology is the science of our immune system, which provides defence against infections, and is often divided into innate immunity and adaptive immunity \(^1\). Innate immunity is the first line of defence against infections with microbial pathogens and every time an infectious agent is encountered the response is similar. It consists of many interacting systems, including epithelial barriers, antimicrobial peptides and effector cells. Distinct patterns on the pathogen are recognized by cells like neutrophils, monocytes, dendritic cells (DC) and natural killer cells (NK cells), clearing the infection by the mechanism of phagocytosis and by secreting inflammatory mediators.

If the innate immune system is bypassed or overwhelmed by the pathogens, the adaptive immune response is required. The adaptive immune response provides a specific response, with an enormous ability to recognize different antigens, and the responsiveness is improved on repeated exposure, providing immunological memory. The cells involved are antigen-specific T and B cells which, when activated, proliferate and develop into effector cells secreting cytokines and antibodies, respectively \(^1\). T cells can be divided into two different populations based on their expression of the cell surface proteins cluster of differentiation (CD) 4 or CD8. \(^1\) B cells differentiates into antibody producing plasma cells when activated by an antigen via its B cell receptor \(^1\).

*Cytotoxic T cells*

Some intracellular bacteria and all viruses infecting different cell types multiply in the cytoplasm and once they have entered the cells they are not susceptible for extracellular antibodies. These pathogens can only be eliminated by the
destruction of the host cell, which is mediated by cytotoxic T cells (T_C).
Cytotoxic T cells are CD8^+ T cells recognizing human leukocyte antigen (HLA) class I molecules expressed on all nucleated cells. The HLA class I express fragments from the infectious pathogens activating the T_C cell which induces cell death by the release of lytic enzymes or by introducing apoptosis .

**Helper T cells**

Helper T cells (T_H) are CD4^+ T cells recognizing HLA class II molecules expressed on professional antigen presenting cells (APC), i.e. DCs, macrophages and B cells. Helper T cells differentiate into different subtypes depending on released mediators from the APC. The APC recognize distinct patterns among pathogens, inducing the production of mediators resulting in differentiation of naïve T cells into T_H1, T_H2 or T_H17 cells. These subtypes differ in the cytokines patterns (Figure 1) and thereby also in function.

![Figure 1 Schematic overview of the possible differentiation factors for T helper (T_H) cells](image-url)
B cells and antibodies

Antigen bound to the B cell receptor is internalized and presented upon HLA class II for T_h cells. Signals from the bound antigen and from the T_h cell induce the B cell to proliferate and differentiate into plasma cells, secreting antigen specific antibodies. The main function of the antibodies are to neutralize pathogens, binding the pathogen for enable phagocytosis or complement activation. The antibodies can be divided into five major isotypes: immunoglobulin M (IgM), IgD, IgA, IgE and IgG.

The immunological synapse

A central event in the development of the adaptive immunity is the activation of T cells. The initiation of this process is the triggering of T cells by APC. The contact molecules between T cells and APC form the so called “immunological synapse”, a place for signalling.

The APC degrades foreign pathogens into peptides, which are expressed together with HLA molecules on the APC surface. A T cell with a T cell receptor (TCR) specific for the presented antigen will bind to the HLA/peptide complex, the CD8 or CD4 binds to the HLA class I or II molecules, respectively, strengthening HLA/TCR interaction, and the T cell will receive its first signal for activation. This will lead to the rearrangement of receptors into a central supra-molecular activation cluster (cSMAC), enriched with TCRs, CD28 and CD2 molecules, surrounded by a peripheral SMAC (pSMAC), enriched with adhesion molecules. CD28 binds to CD80 and CD86 expressed on the APC, generating a signal lowering the threshold for T cell activation. The binding of adhesion molecules will extend the contact between APC and T cells, mediating a longer HLA/TCR interaction. Efficient T cell activation requires one signal from the HLA/TCR complex and second signals from the
co-stimulatory (Figure 2) and adhesion molecules present in the cSMAC and pSMAC.

**Figure 2** A schematic overview of the immunological synapse. The antigen presenting cell (APC) presents a degraded protein as peptides, bound to human leukocyte antigen (HLA) molecules. The HLA/p peptide complex is recognized by the T cell receptor (TCR) and delivers the first signal for T cell activation via CD3. The T cell also receives a second signal though the binding of CD28-CD80/86. Once activated, cytotoxic T lymphocyte associated antigen (CTLA-4) is up-regulated and, binding to CD80/86, confers an inhibitory effect.

**Down-regulation of response**

Once a successful T cell activation has occurred and the pathogen is rendered harmless, the immune response must be down regulated to avoid damage in the surrounding tissue. The mechanisms in terminating a lymphocyte response include T cell inhibition by cytotoxic T lymphocyte associated antigen (CTLA-4, CD152), activation-induced cell death or down regulation mediated by regulatory T cells.⁶
Cytotoxic T lymphocyte associated antigen-4 (CD152) is primarily an intracellular membrane protein and it is upregulated on the T cell surface during activation \(^7,^8\). The peak of messenger RNA (mRNA) expression appears after six to 24 hours and maximal protein expression two to three days after initial T cell activation \(^7,^8\). The ligands for CTLA-4 are the same as for CD28 (Figure 2), but the affinity of CTLA-4 for both CD80 and CD86 is between 20- and 100-fold higher than that of CD28, with a strong bias towards CD80 \(^9\). Several mechanisms have been proposed for the down-regulation of immune response by CTLA-4, including competition with CD28 for ligands and/or interference with intracellular signalling pathways for TCR and/or CD28 signalling, thereby inhibiting proliferation and interleukin (IL)-2 secretion by T cells \(^10,^11\). Cytotoxic T lymphocyte associated antigen-4 may also be involved in disassociation of the cSMAC \(^12\) or lead to a more transient interaction between T cells and APC, diminishing T cell activation \(^13\).

Regulatory T cells have been proposed to have a role in suppression of immune response and maintenance of self-tolerance \(^14,^15\). Both inducible and naturally regulatory T cells (iTreg and nTreg, respectively) are involved, but these populations differ in their mechanisms of action. Inducible regulatory T cells develop in the periphery from conventional CD4\(^+\) T cells under the influence of cytokines and are defined by their secretion of IL-10 (regulatory T cells type 1 (Tr1)) and transforming growth factor (TGF)-\(\beta\) (T\(\beta\)3) \(^14-17\). Suppression by naturally occurring CD4\(^+\)CD25\(^+\) T cells is mediated by cell contact-dependent mechanisms, e.g. by “outside-in” through CD80/86, inhibiting IL-2 production in responder cells \(^18-20\) or by the secretion of IL-35 \(^21\).

In conclusion, the immune system defends us against infections in different ways, adapting its response depending on type of pathogen. The innate immunity serves as the first line of defence and the adaptive immunity serves a more
specific response. The down regulation of the immune response is important in suppressing activated cells after pathogen clearance and preventing tissue damage.

**T helper cells**

The T\(_H\)1 and T\(_H\)2 cells were originally characterized in mice \(^{22}\) and later also in humans \(^{23}\). Murine and human T cells produce cytokines in similar patterns, with T\(_H\)1 cells producing IL-2, interferon (IFN) -\(\gamma\) and tumor necrosis factor (TNF)-\(\beta\), whereas T\(_H\)2 cells produce IL-4, IL-5, IL-9 and IL-13 (Figure 1). T\(_H\)1 and T\(_H\)2 cells play different roles in the response against invading pathogens. T\(_H\)1 cells are effective in cell-mediated inflammatory reactions, recruiting and activating macrophages and T\(_C\) cells to fight against intracellular infections and stimulating B cells to produce opsonising and neutralizing antibodies. T\(_H\)2 cells are on the other hand involved in the humoral immune response by activating antibody secreting B cells, eosinophils and basophils in the defence against parasites and helminths.

The T\(_H\)1/T\(_H\)2 paradigm is a rather generalized and simplified picture, but this concept has been, and is still today, rather useful. However, recently a subset distinct from T\(_H\)1 and T\(_H\)2 was discovered and named T\(_H\)17 according to the production of the highly pro-inflammatory cytokines of the IL-17 family (Figure 1) \(^{24}\). The receptors for IL-17 are expressed on epithelial cells and other cell types, and IL-17 is therefore believed to promote tissue inflammation. The complete mechanistic function of these T\(_H\)17 cells is however not fully understood.
Cytokines, cytokine receptors and transcription factors of T\(\text{H}\) subtypes

One of the most critical cytokine inducing a T\(\text{H}1\) immune response is IL-12\(^{25}\). Interleukin-12 is a heterodimer composed by the two subunits IL-12\(\alpha\) (p35) and IL-12\(\beta\) (p40) signalling through the IL-12 receptor (IL-12R). The IL-12R is composed of a \(\beta_1\) and a \(\beta_2\) subunit. Both \(\beta_1\) and \(\beta_2\) are necessary for binding IL-12, but the \(\beta_2\) subunit is responsible for transferring the signal\(^{26}\). Interleukin-12 acts on cells of the innate and the adaptive immune system, inducing significant levels of IFN-\(\gamma\). Interferon-\(\gamma\) induces the expression of the transcription factor T-box expressed in T cells (T-bet), via signal transducers and activator of transcription (STAT) 1\(^{27}\). This even further increases the production of IFN-\(\gamma\), but also induces IL-12R\(\beta_2\) chain expression\(^{27-29}\). Interleukin-12 strongly induces STAT4 phosphorylation in human T\(\text{H}1\) cells\(^{30}\), augmenting the IFN-\(\gamma\) production\(^{28}\) as well as increasing the expression of IL-12R\(\beta_2\)\(^{31}\), completing the T\(\text{H}1\) developmental commitment process. Once activated, T-bet, together with IFN-\(\gamma\), forms an auto-regulatory positive feedback loop to maintain a type 1-mediated response\(^{27}\) and inhibit IL-5 production\(^{32}\).

The T\(\text{H}2\) lineage is predominantly induced by the IL-4/STAT6 signalling pathway. Although several types of cells have been shown to secrete IL-4, the initial source of IL-4 is still unknown. Interleukin-4 binds to the IL-4R, composed of the common \(\gamma\) chain and the IL-4R\(\alpha\) chain. The phosphorylated IL-4R can further recruit and activate STAT6, which in turn induces GATA-3 and c-maf\(^{33}\). GATA-3 has been demonstrated to be selectively expressed in T\(\text{H}2\) cells\(^{34}\) and both GATA-3 and c-maf are important transcription factors for IL-5 and IL-4\(^{35,36}\). Retroviral introduction of GATA-3 or STAT6 into T\(\text{H}1\) cells introduced T\(\text{H}2\) specific cytokines and suppressed IFN-\(\gamma\) production, further stressing the importance of GATA-3 in T\(\text{H}2\) development\(^{33,37}\).
The relatively newly discovered T\textsubscript{H}17 cells produce the pro-inflammatory cytokines IL-17A, IL-17F, IL-21, IL-22 and IL-6\textsuperscript{24,38-40}. T helper 17 cells were previously believed to develop from naïve T cells in response to IL-23\textsuperscript{41-44}. Later TGF-β and IL-6 were shown to elicit T\textsubscript{H}17 cells in mice\textsuperscript{45} while IL-23 was found to retain the T\textsubscript{H}17 commitment. Recent data suggest that TGF-β together with pro-inflammatory cytokines like IL-6 and IL-1β is necessary also in humans for the up-regulation of the transcription factor RORc (the human ortholog of mouse ROR\textgreek{t})\textsuperscript{40,46}, which is required for the induction of IL-17 production\textsuperscript{40,47}.

Figure 3 summarize the important factors involved in the differentiation of naïve T\textsubscript{H} cells into different subtypes.

**Naturally occurring regulatory T cells**

Naturally occurring regulatory T cells is a subpopulation of T\textsubscript{H} cells with low proliferative capacity and is specialized in down regulation of the immune response. Naturally regulatory T cells develop in thymus and are found in the peripheral lymphoid tissue, where the induction of suppressor capacity requires activation through TCR, but once activated their function can be nonspecific. Suppression by nTreg cells is mediated by cell contact-dependent mechanisms, e.g. by “outside-in” through CD80/86, by inhibiting IL-2 production in responder cells\textsuperscript{18-20}, or by the secretion of IL-35\textsuperscript{21}.

Naturally occurring regulatory T cells have been characterized by the expression of CD25 (IL2Ra)\textsuperscript{48,49}, CTLA-4\textsuperscript{50,51}, glucocorticoid-induced TNF receptor (GITR)\textsuperscript{52} and inducible co-stimulator (ICOS)\textsuperscript{53} together with the absence of CD127\textsuperscript{54}.
The forkhead/winged helix transcription factor Foxp3 is the transcription factor for nTreg and IL-2 is an important cytokine for maintaining Foxp3 expression, signaling through STAT5. Transforming growth factor-β also supports the maintenance of Foxp3. The importance of Foxp3 in nTreg function has been demonstrated in mice and humans with mutations in the Foxp3 gene. These mutations are in humans responsible for a disease called immunodysfunction polyendocrinopathy enteropathy X-linked syndrome (IPEX), causing an impairment of nTreg cells. This disease is associated with several autoimmune diseases, such as type 1 diabetes (T1D).

Recent studies have shown Foxp3 expression in activated CD4⁺CD25⁻ T effector cells. The Foxp3 expression is making them hyporesponsive, suggesting that Foxp3 induction turn of T cell activation. Stimulation of effector T cells, resulting in a transient up-regulation of Foxp3, does not result in suppressive activity, however. For the conversion of effector T cells to regulatory T cells, a high and stable expression of Foxp3 is required. The function of Foxp3 in effector T cells and the molecular factors that regulate its expression remains to be elucidated. However, the transcription factor nuclear factor of activated T cells (NFAT)-c2, together with STAT5, have been found to be involved in Foxp3 up-regulation.

Figure 3 summarize the important factors involved in the differentiation and function of nTreg cells.

In conclusion, the fate of T<sub>H</sub> lymphocytes is controlled by antigen receptors and secreted cytokines from APCs, influencing the development of different subtypes. The nTreg is important in controlling the action of activated effector T cells, although the mechanisms are not fully understood.
Figure 3 Schematic picture of T\(_H1\), T\(_H2\), T\(_H17\) and nTreg cells together with cytokines, receptors and transcription factors important for their development. Markers studied in Paper II are indicated in bold.
Introduction to diabetes mellitus

Definition and diagnosis of diabetes

Diabetes mellitus comprises a group of metabolic disorders characterized by a chronic hyperglycaemia caused by defects in insulin secretion, insulin action or both \(^6\). The effects of the chronic hyperglycaemia in diabetes are damage, dysfunction and failure of several organs, in particular eyes, kidneys, nerves, blood vessels and heart.

The symptoms associated with diabetes are caused by the hyperglycaemia, including thirst, polyuria, blurring of vision and weight loss \(^6\). These symptoms are often not severe and may even be absent, leading to pathological and functional changes long before diagnosis is made \(^6\). Diabetic children are often presenting severe symptoms at onset with very high blood glucose levels, glycosuria and ketonuria \(^6\).

The diagnosis is based on measurements of plasma/blood glucose in combination with the symptoms mentioned above. If a person is asymptomatic, the diagnosis should be made only after repeated plasma/blood glucose tests where the value should be in the diabetic range, or the diagnostic criteria for diabetes is met in the oral glucose tolerance test (OGTT) \(^6\). The diagnostic criteria are the same in children and adults but in most children the diagnosis is made by plasma/blood glucose test, without an OGTT \(^6\), since the symptoms are severe. The diagnosis can be confirmed by plasma glucose value \(\geq 12.2\) mmol/L or fasting plasma glucose \(\geq 7.0\) mmol/L. The diagnostic criterion for fasting whole blood glucose is \(\geq 6.1\) mmol/L. If the diagnosis still is uncertain, an OGTT is used as confirmation of the diagnosis. In children a plasma glucose value \(\geq 11.1\) mmol/L in capillary blood or \(\geq 10.0\) mmol/L in venous blood are
considered as diagnostic cut-off values for diabetes two hours after an oral glucose load of 1.75 g per kg body weight.

**Classification of diabetes mellitus**

According to the World Health Organization (WHO), diabetes mellitus can be divided into four groups depending on aetiology.

**Type 1 diabetes** is due to pancreatic islet β-cell destruction \(^{67}\) and thus “insulin is required for survival, to prevent the development of ketoacidosis, coma and death” \(^{67}\). At least ten percent of all cases of diabetes are T1D. Most cases are autoimmune, characterized by the presence of autoantibodies against antigens present in the pancreatic islets i.e. insulin, glutamic acid decarboxylase (GAD\(_{65}\)) and/or tyrosine phosphatase-like insulinoma antigen-2 (IA-2), identifying an ongoing autoimmune process. Insulin dependent T1D without signs of autoimmunity, where no autoantibodies are found but β-cell destruction is evident, is classified as a type 1 idiopathic diabetes \(^{67}\). In children the disease process usually is rapid, but more gradual in adults. The slowly progressive form among adults, with phenotypic type 2 diabetes (T2D) but a slowly developing autoimmune process, is referred to as latent autoimmune diabetes in adults (LADA).

**Type 2 diabetes** is the most common type of diabetes, including the forms caused by defects in insulin secretion and/or insulin resistance. Exogenous insulin is usually not required for survival \(^{67}\) and the treatment consist of diet, increased physical activity and weight loss. Type 2 diabetes is still rare in children and adolescents in Sweden, but is becoming increasingly common in e.g. USA \(^{68}\). The symptoms of the hyperglycaemia are usually not severe, but the disease increases the risk of severe late complications.
**Gestational diabetes** means pronounced carbohydrate intolerance with onset during pregnancy resulting in hyperglycaemia 67.

**Other specific types** include less common types where the cause can be identified in a relatively specific manner 67. This group of diabetes includes for example genetic defects on β-cell function or insulin action, e.g. different forms of maturity onset diabetes in the young (MODY). There are also rare cases of diabetes caused by diseases of exocrine pancreas, drug- or chemical induced or caused by infections 69.

**Epidemiology of type 1 diabetes**

The incidence of T1D is increasing all over the world, with significant variation in the incidence in different parts of the world. The highest incidence is seen among Caucasians and the lowest in Asia and South America. The WHO has tried to estimate the incidence of T1D in 57 countries around the world during a 10-year period, between 1990-1999 70, despite the difficulties in several countries with high child mortality. The incidence rates varied between 0.1/100,000 per year in China and Venezuela to 40.9/100,000 in Finland 70, where Finland had a peak of 48.5 cases/100,000 in 1998 71. Sweden had an average incidence of 30.0/100,000 per year during 1990-1999 70. Since then, there has been a steady increase in incidence up to >60/100 000 in Finland 72 and >40/100 000 in Sweden, according to the national Swedish registry (personal communication).

The mean annual increase worldwide between 1995-1999 was 3.4% and the corresponding figure for Sweden between 1990-1999 was 3.6% 70. The increasing incidence of T1D in Swedish children may perhaps to some extent be explained by a shift to a younger age at diagnosis 73.
Aetiology of type 1 diabetes

The aetiology of T1D is largely unknown but the development is believed to be determined by a combination of genetic susceptibility genes, immune dysregulation and environmental factors. The resulting autoimmune process may be initiated several years before clinical onset of T1D (Figure 4). The autoimmune process causes a decrease in β-cell mass, leading to diminishing insulin production. Several genetic susceptibility loci have been proposed to be involved in the development of T1D \(^74\) and a number of environmental factors have been suggested to additional contribution \(^75\).

![Figure 4](image_url)

**Figure 4** Schematic illustration over T1D development. Interactions between genes, the immune system and environmental factors may trigger an autoimmune response leading to the loss of β-cell mass and the progression to T1D (Adapted from Atkinson & Eisenbarth \(^76\)).

In conclusion, T1D is a serious disease, dependent of lifelong support of insulin and the incidence of T1D in Sweden is among the highest in the world and is still increasing. The mechanisms behind the development are still largely unknown, but there seems to be interactions between several parameters.
Pathogenesis of type 1 diabetes

Genetic risk of type 1 diabetes

Several genes are involved in the pathogenesis of T1D. These genes are neither necessary nor sufficient for disease to develop, but they modify the risk. The HLA is the major locus associated with T1D\textsuperscript{74, 77, 78}, but also CTLA-4\textsuperscript{74, 79, 80}, as well as other loci\textsuperscript{74, 80-82} have been found to modulate the susceptibility.

Human leukocyte antigen

The first loci identified to increase the risk for T1D was in the HLA region\textsuperscript{83}. This region is located on chromosome 6p21 and has been implicated as a major genetic risk factor. The genes in this area are arranged in three sub-regions, class I, II and III, where class II has the major influence on T1D risk. HLA class II-DQ alleles have the strongest genetic association with T1D\textsuperscript{84}, but DR alleles have the ability to modify the risk conferred by the DQ locus, suggesting that both DR and DQ are likely to be important for determining the risk for T1D\textsuperscript{85, 86}. Each HLA molecule consists of one alpha (A) and one beta (B) chain, which together form a heterodimer (Figure 5). These chains of DQ are encoded by the DQA1 and DQB1 genes, some of which are in linkage disequilibrium with each other. This means that these alleles are showing a non-random association with each other, whereby it is possible to deduce a DQA1 allele based on the DQB1 and vice versa.

An asterisk is used to separate the gene name from the alleles and the alleles for each gene are numbered using four digits. The first two describe the most closely associated serologic specificity and the latter two describe the subtypes within this serologic specificity. Different DR/DQ molecules might have varying degrees of binding affinity to specific peptides, but as the binding affinity of a given DR or DQ molecule to a particular epitope is identical in all
ethnic groups, the effect of the same HLA molecule on T1D risk should not vary across ethnic groups if the primary auto-antigens are identical.

**Figure 5** The HLA class II molecule.

Most studies regarding genetic risk factors are done in Caucasian populations and HLA allele and haplotypes frequencies vary considerably across ethnic groups\(^8^7\). For instance, Asian populations show much lower frequencies of the T1D high risk DR-DQ haplotypes relatively common among Caucasian populations; and the disease prevalence in these two ethnic groups might mirror these differences. Certain HLA class II haplotypes show strong associations with the development of T1D and are found more often among T1D children than controls. Others are more often found among controls than T1D children and are then considered to be protective (Table 1).

<table>
<thead>
<tr>
<th>Risk of T1D</th>
<th>DR</th>
<th>DQA1</th>
<th>DQB1</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>*0401/2/4/5</td>
<td>*0301</td>
<td>*0302</td>
<td>DR4-DQ8</td>
</tr>
<tr>
<td>High</td>
<td>*03</td>
<td>*05</td>
<td>*02</td>
<td>DR3-DQ2</td>
</tr>
<tr>
<td>Neutral</td>
<td>*0101</td>
<td>*0101</td>
<td>*0501</td>
<td>DR1-DQ5</td>
</tr>
<tr>
<td>Protective</td>
<td>*1501</td>
<td>*0102</td>
<td>*0602</td>
<td>DR2-DQ6</td>
</tr>
<tr>
<td>Protective</td>
<td>*05</td>
<td>*05</td>
<td>*0301</td>
<td>DR5-DQ7</td>
</tr>
</tbody>
</table>
The HLA class II is responsible for the presentation of antigenic peptides to T cells. Appearance of β-cell autoantibodies have been associated with HLA haplotypes connected with T1D \(^{88,89}\) and aberrant cytokine responses have been reported in association with HLA class II risk haplotypes \(^{90-92}\). Thus, peptide presentation may affect T1D susceptibility.

**Cytotoxic T lymphocyte associated antigen 4**

Cytotoxic T lymphocyte associated antigen-4 is an important regulator of the immune system as it, when interacting with its ligands CD80/86, is responsible for the down-regulation of T cell activation. The gene of CTLA-4 is located on chromosome 2q33 \(^{93}\) and consist of four different exons where several single nucleotide polymorphisms (SNP) have been identified \(^{79,80,94}\). The CTLA-4 polymorphism + 49 A/G (rs231775), where a shift from an A to an G results in an amino acid substitution, is the SNP most associated with autoimmune disease, but also other SNPs in the gene of CTLA-4, like CT60 (+6230) A/G (rs3087243) and CTBC217_1 C/T (rs2033171), are related to the development of autoimmune diseases \(^{80,94-98}\). Relatively little is known about the effect of these susceptibility polymorphisms.

The +49 A/G polymorphism has been associated with an altered function of CTLA-4, resulting in a higher cytokine secretion and proliferative response upon T cell activation \(^{99,100}\), perhaps due to a reduced up-regulation of CTLA-4 in the individuals with susceptibility SNP \(^{101}\).

The CT60 SNP has also been associated with disease susceptibility and may correlate to a lower mRNA of the soluble form of CTLA-4 \(^{80,102}\), although this has not been confirmed by others \(^{97}\). The CT60 susceptibility SNP has also been shown to alter the signalling threshold of CD4\(^+\) T cells \(^{103}\).
**Inducible co-stimulator**

The ICOS is a co-stimulatory molecule induced on T cells during activation, enhancing T cell responses to foreign antigens and induces the production of several cytokines, including IL-4, IL-5, IFN-γ and IL-10, from T cells\(^{104}\). The ICOS gene is mapped to the same region as CTLA-4 on chromosome 2q33 and the polymorphism in the ICOS gene may contribute to disease susceptibility too. Several SNPs have been found in the ICOS gene\(^ {105}\). The CTIC154_1C/T (c.1624, rs10932037) is a SNP located in the 3′ UTR of ICOS gene\(^ {80}\) and individuals homozygous for the CTIC154_1 C/C have higher expression of ICOS than heterozygous individuals\(^ {102}\).

**Lymphoid tyrosine phosphatase**

The gene of PTPN22, encoding the protein Lymphoid Tyrosine Phosphatase (LYP), is located on chromosome 1p13 and has been implicated in the pathogenesis of T1D\(^ {81, \ 82, \ 106}\). Lymphoid Tyrosine Phosphatase is a protein found in the cytosol and nucleus of haemapoetic cells and is a strong negative regulator of T cell activation. For T cells to become activated several proteins inside the cells are phosphorylated and LYP is causing dephosphorylation of these proteins, e.g. the TCR/CD3 complex, thereby inhibiting T cell activation\(^ {107}\). A SNP has been identified within PTPN22, the gene encoding LYP, at position 1858(rs2476601), where a shift from a C to a T results in a replacement of an amino acid\(^ {81}\). Individuals homozygous for 1858T/T have been shown to have a defect in TCR response, which results in a gain of inhibitory function\(^ {108}\), i.e. dephosphorylation of the signalling proteins occurs much more efficiently. The variant of PTPN22 1858T has been associated with an increased risk for autoimmune diseases, including T1D\(^ {81, \ 82, \ 106}\). One hypothesis postulated is that 1858T allele predisposes to autoimmune disease due to a more active
suppression of TCR signalling during thymic development, allowing survival of autoreactive T cells that normally would be deleted \textsuperscript{107}.

**In conclusion, several genetic factors are important regulators of the immunological response, also making them important in the pathogenesis of T1D. More detailed studies are required to understand the mechanisms underlying these effects, however.**

**Autoantibodies against β-cell antigens**

Before clinical manifestation of T1D, autoantibodies against β-cell antigens can be detected in most individuals. These autoantibodies can be used as markers of the autoimmune process and may be present years before clinical T1D. The presence of multiple autoantibodies can be used to identify individuals at risk of T1D \textsuperscript{109}. Today, three major islet autoantibodies are used for prediction of T1D.

**Insulin**

Insulin was the first β-cell antigen detected in newly diagnosed T1D patients \textsuperscript{110} and is the only β-cell specific auto antigen. Insulin autoantibodies (IAA) are often the first autoantibody to appear in individuals who develop autoimmunity to islet antigens and the levels of IAA correlate inversely with age since levels of IAA decline at older ages \textsuperscript{111}. The frequency of IAA positive patients seems to be more common in children from areas with high incidence of T1D and the presence of IAA appears to give a clinically milder disease \textsuperscript{112}.

Insulin autoantibodies are found in 40-70\% of newly diagnosed patients \textsuperscript{112-114} and in 0.9-3\% of a healthy population \textsuperscript{115,116}. 
Glutamic acid decarboxylase

Glutamic acid decarboxylase was originally detected as a 64 kilo Dalton (kDa) protein from islet homogenates to which sera from newly diagnosed T1D patients were shown to immunoprecipitate \[^{117}\]. These autoantibodies were also found before clinical onset of T1D \[^{118}\]. Later, this 64 kDa protein was identified as the enzyme GAD and was found to be expressed in pancreatic islet cells and in peripheral nerves \[^{119}\]. In terms of action, GAD is involved in the conversion of glutamic acid to GABA, an inhibitory neurotransmitter, but the function in the pancreatic islets is however not clear \[^{120}\]. There are two isoforms of GAD, GAD\(_{65}\) and GAD\(_{67}\), but only GAD\(_{65}\) has been shown to be expressed in human islets and is responsible for the autoantibody response \[^{121}\].

Autoantibodies against GAD (GADA) are found in about 50-80% of newly diagnosed T1D patients \[^{112, 113, 120, 122}\]. In contrast 0-3% of the general population has GADA \[^{115, 116, 120, 123}\].

Tyrosine phosphatase–like insulinoma antigen-2

Besides the 64 kDa protein identified as GAD, sera from T1D patients were found to bind another protein that is cleaved into a 37 kDa and 40 kDa fragments by trypsin. The 40 kDa and 37 kDa proteins were subsequently identified as the proteins IA-2/ICA512 and IA-2β, respectively \[^{124-127}\]. Both IA-2 and IA-2β are transmembrane proteins and autoantibodies to have been shown to be directed against the intracellular part of the IA-2 protein (IA-2ic) \[^{124, 128}\] where 95% of T1D patients with autoantibodies against IA-2 react with the carboxyl-terminus (amino acids 771-979) \[^{120}\].

About 55-80% of newly diagnosed T1D patients have autoantibodies against IA-2 (IA-2A) \[^{113, 120}\] while 0-2.5% of the general population has IA-2A \[^{115, 116, 120, 123}\].
In conclusion, the emergence of autoantibodies against insulin, GAD and IA-2 are markers for an ongoing autoimmune process and can be used to identify individuals at risk of T1D.

**Environmental factors**

The genetic predisposition can not entirely explain the development of T1D in certain individuals, since there is approximately 40-50% concordance of developing autoantibodies or T1D in identical twins \(^{78, 129, 130}\) and only about six to 20% of siblings to probands are affected \(^{131-133}\). Furthermore the rapidly increasing incidence is unlikely to depend on changes of genetic predispositions in the population but may be a consequence from environmental changes. Several environmental factors have been suggested to trigger the autoimmune response and the development of T1D and some of them are presented more in details below.

**Viral infections**

Viral infections have been suggested as risk factors for the development of T1D \(^{134-140}\), and several mechanisms how viruses can trigger autoimmunity have been proposed \(^{141}\). Enteroviruses, and in particular Coxsackie virus B4 (CVB4), have been proposed as triggers of β-cell autoimmunity and T1D \(^{142, 143}\). Enteroviral infections during pregnancy have been associated with increased risk for T1D in the offspring \(^{134, 135}\), although discrepant results exist \(^{143-145}\). Also, increased numbers of enterovirus infections have been demonstrated in children later progressing to T1D \(^{134}\). Children with T1D have, compared to healthy children, also been shown to have an impaired immune response against CVB4 \(^{146, 147}\).

The hygiene hypothesis suggest that increased hygiene may cause changes in the gut bacterial flora, influencing the maturation of the immune system, facilitating
imbalance and thereby autoimmune reactions in genetically predisposed individuals \(^{148}\) and thus the increased hygiene might lead to low immunity against certain viruses. This fits with the idea that low frequencies of enteroviral infections increase the susceptibility of diabetogenic effect of enteroviruses \(^{149}\).

**Cow’s milk**

Associations between cow’s milk exposure and β-cell autoimmunity and later T1D development have been shown in several studies \(^{150,151}\), even though contradicting results exist \(^{152}\). Early introduction of cow’s milk based formula and high consumption of cow’s milk have been associated with higher levels of β-cell autoantibodies \(^{153}\). An important question is what component in cow’s milk that can trigger β-cell autoimmunity and several suggestions have been made, including different cow milk proteins and particularly bovine insulin. Enhanced immune response to cow milk proteins have been shown in children later developing T1D \(^{154}\) suggesting a primary failure in the gut immune system in children at risk of T1D. This could result in aberrant immune response to dietary proteins, such as bovine insulin. Early exposure to bovine insulin can enhance the humoral and cellular immune response to insulin in infants \(^{155}\), and thus be the link between β-cell autoimmunity and cow’s milk.

**Gluten**

Celiac disease is a chronic inflammation in the small intestine resulting in villus atrophy and this inflammation is triggered by wheat gluten. The association of celiac disease and T1D have been shown in several studies \(^{156,157}\), possibly partially explained by the shared genetic background of DQA1*05-DQB1*02 (DR3-DQ2) \(^{158}\). Early introduction of gluten has been reported as a risk factor for β-cell autoimmunity \(^{159}\), but the mechanisms mediating the risk are not understood.
**β-cell stress**

Increased weight gain in infancy \(^75, 160\) and psychological stress \(^{161}\) may induce extra pressure on the β-cells and can be regarded as risk factors for the development of T1D. During infancy and puberty, periods of rapid growth, the need for insulin increases, causing β-cells work hard to produce the required insulin. The increased insulin production may result in stimulation of the autoimmune process leading to overt T1D \(^{148}\). Psychosocial stress in families may also affect insulin secretion and insulin sensitivity, although the biological mechanisms are still not known. Several psychological factors can influence the development of autoantibodies as high parental stress and serious life events can induce IA-2A in one or two and a half-year old children of a general population, respectively \(^{161, 162}\).

*In conclusion, an immunological imbalance may result in multiple environmental factors triggering the development of β-cell autoimmunity and T1D, although the detailed mechanisms are unknown.*

**T cells and cytokines in the pathogenesis of type 1 diabetes**

Clinical, autoimmune diabetes is caused by a loss of immunological tolerance, causing a destruction of the insulin producing β-cells in pancreas, called insulitis. The initial event triggering this autoimmune reaction is still unknown, however the immune reaction to β-cells can be divided into three stages \(^{163}\). The first stage is the primary sensitization against β-cell antigens, the second stage is APC presentation of auto-antigens causing an immune reaction and consequent inflammation in the islet. The third and last stage includes a cycle of harmful interactions between immune cells and β-cells.
Autoimmune insulitis has been investigated in several animal models like the nonobese diabetic (NOD) mice and BioBreeding (BB) rats, greatly enhancing our knowledge in pathogenesis of T1D. Activated T cells, specific for β-cell auto-antigens, home to the pancreas, expand clonally and start an autoimmune reaction culminating in β-cell destruction. The release of cytokines from T\textsubscript{H} cells recruit macrophages into the tissue, activating them, eliminating the antigen-bearing cell. The cytokines also activate T\textsubscript{C} cells to destroy target cells expressing the antigen presented together with major histocompability complex (MHC) class I and NK cells to destroy target cells independently of MHC expression. Also other mediators released, like free radicals and nitrix oxide, support the destruction since they are highly toxic to the β-cell.

In human insulitis, immunostaining has revealed infiltrating CD\textsuperscript{4}, CD\textsuperscript{8} T cells as well as macrophages and the role of cytokines have been investigated in several studies, demonstrating discrepant results. The model of T\textsubscript{H}1 and T\textsubscript{H}2, and subsequently IFN-γ and IL-4, has been used in several studies exploring the possible changes in immune response in T1D. Peripheral blood mononuclear cells (PBMC) have been used for these studies and the expression and secretion of various cytokines have been explored in unstimulated PBMC as well as after various stimulations. Peripheral blood mononuclear cells been shown to produce higher amounts of the cytokine IFN-γ as well as lower levels of IL-4. However, others have shown a lower expression and secretion of IFN-γ, while the level of IL-4 was similar in T1D patients and controls. This low secretion of IFN-γ has also been shown in individuals at high risk of developing T1D after stimulation with the three auto-antigens GAD, insulin and IA-2 or with the viral antigen CVB4.

In conclusion, the pathogenesis of T1D is believed to result from cytokine and immunoregulatory imbalance, culminating in β-cell death and subsequent T1D.
AIM OF THE THESIS

The general aim of this thesis was to investigate how diverse environmental and immunological risk factors associated with the development of T1D influence the immunological system.

The specific aims of the individual papers were:

I. To study associations between environmental risk factors and T1D-related autoantibodies at one year of age in a general population.

II. To investigate the profile of T helper cell polarization markers in peripheral blood, both in children with increased risk of T1D, either genetic risk and/or with diabetes related autoantibodies, and in children with T1D during the first year after manifestation of T1D.

III. To examine if children with T1D associated gene polymorphisms, such as HLA class II risk alleles or the CTLA-4 +49 A/G, CTLA-4 CT60 A/G and CTLA-4 CTBC217_1 C/T polymorphism, are associated with an aberrant cytokine responses to diverse antigens.

IV. To study the amount and fluctuation of CD4⁺CD25\textsuperscript{high} regulatory T cells during 18 months period from the manifestation of T1D and also to compare the function of CD4⁺CD25\textsuperscript{high} regulatory T cells in children with T1D and healthy children.
SUBJECTS & METHODS

Study populations

Three different study populations were used: healthy children, healthy children with increased risk for T1D and children with manifest T1D.

Healthy school children

Healthy school children were recruited from Rydsskolan in Linköping, Sweden. The children were asked to fill out a form together with their parents. This form contained questions about atopic status (eczema, hay fever, asthma or usage of allergy medicines), presence of type 1 or type 2 diabetes, thyroiditis, rheumatoid arthritis or celiac disease in the children or in any first degree relative. Children with any of the conditions above or with first degree relatives with any of the conditions above were excluded. In Paper II we studied samples from eight children with the mean age of 11.7 years (range 11.0-12.8 years) and in Paper IV we studied samples from 17 children with the mean age of 11.7 years (range 5.3-16.3 years).

The ABIS study

In Paper I, II and III we studied samples taken from children participating in the ABIS study (All Babies in south-east Sweden) which is a population-based prospective cohort study of 17 055 (out of 21 700 invited) infants born between 1st October 1997 and 1st October 1999. The aim of the study was to study the importance of environmental factors for the development of T1D, but also of other autoimmune diseases and allergy in the general population. A second aim was to identify children with risk of developing T1D, and if effective intervention appeared, to be able to intervene to prevent some cases of the disease.
Subjects & Methods

The newborns have been followed from birth up to five years of age with biological samples and questionnaires. Biological samples have been collected at birth (cord blood, breast milk, hair from mothers), one (blood, stool and hair), two and a half and five years of age (blood, stool, urine and hair). At the age of five, venous blood samples were taken, enabling analysis of cell mediated immunity.

In Paper I we studied capillary blood samples from 6,000 children taken at one year of age together with at-birth and one year questionnaires. Autoantibodies to GAD and IA-2 were analyzed in 5,765 and 5,635 children, respectively. In Paper II we studied venous blood samples from 44 children taken at five years of age. These children were selected based on their risk for T1D. Fifteen children as they had T1D associated HLA class II risk haplotypes DQA1*05-DQB1*02 (DR3-DQ2) and/or DRB1*0401/2/4/5-DQB1*0302 (DR4-DQ8), 13 children because of their autoantibody positivity for GADA and/or IA-2A and/or IAA and finally, for comparison, 16 children without any HLA risk haplotypes or autoantibodies. In Paper III we used randomly collected venous blood samples from 67 children taken at five years of age.

Type 1 diabetic patients

In Paper II and IV we studied samples taken from children with manifest T1D. All these children have been recruited at the Pediatric Clinic at Linköping University hospital and the diagnosis was based on the WHO criteria from 1999.

In Paper II, samples from 17 children were taken at three follow-up visits, ten days after diagnosis, one to three months after diagnosis and nine to 18 months after diagnosis. Mean age at diagnosis was 10.4 years (range 6.0-15.6 years). In Paper IV, samples were taken from 20 children at four follow-up visits, ten 

42
days after diagnosis, three month after diagnosis, nine month after diagnosis and 18 month after diagnosis. Mean age at diagnosis was 10.9 years (range 3.5-16.8 years).

**Methods**

*Measurement of autoantibodies*

Autoantibodies against GAD, IA-2 and insulin were analyzed by radiobinding assay. Capillary blood and serum samples were taken at well-baby clinics and stored in freezers at -20°C until analysis. Capillary whole blood samples were analyzed in Paper I and serum samples in Paper II and III. Serum samples in Paper II and III were also analyzed for IAA.

The complementary DNA (cDNA) for GAD$_{65}$ and IA-2ic was extracted from plasmid-carrying *E.Coli* (a kind gift from Professor Å. Lernmark, Seattle) and transcribed and translated in vitro in the presence of $^{35}$S-labeled methionine. All samples analyzed for GADA and IA-2A were incubated with $^{35}$S-labeled protein where after IgG antibodies were precipitated before washing and measurement of radioactivity in a Microbeta Tri-Lux (Wallac, Turku, Finland). The results were expressed as concentrations of autoantibodies, calculated in relation to a standard curve. Sample with values above the highest standard were diluted to fit the standard curve. The sensitivity limits for quantitative determinations were 7.8-500 units/mL for GADA and 4-176 units/mL for IA-2. Background counts were subtracted from each well and duplicate samples with a coefficient of variation (CV) over 20% were re-run.

The samples analyzed for IAA were tested in competition assay with equal volumes of human recombinant unlabeled insulin (Sigma-Aldrich, Stockholm, Sweden) and human recombinant insulin labelled with $^{125}$I (3-
Subjects & Methods

$[^{125}\text{I}]$iodo[tyrosyl]$_{A14}$ insulin) (Amersham Biosciences). The samples were incubated with recombinant insulin, before precipitation, washing and measurement of radioactivity in a gamma counter. The results were expressed as concentrations of IAA, calculated in relation to a standard curve. Sample with values above the highest standard were diluted to fit the standard curve. The sensitivity limits for quantitative determinations were 1.96-125 units/mL. Background counts were subtracted and specific counts were calculated by subtraction of counts of excess unlabeled insulin from counts of labelled insulin. Duplicate samples with a CV over 20% were rerun.

In Paper I we used both the 99$^{th}$ percentile and the 90$^{th}$ percentile as cut-off for autoantibody positivity. The 99$^{th}$ percentile corresponded in our study population to 37.2 WHO units for GADA and 32.7 WHO units for IA-2A. The 90$^{th}$ percentile corresponded in our study population to 30.9 WHO units for GADA and 29.9 WHO units for IA-2A. In Paper II and III, the 98$^{th}$ percentile was the cut-off for positivity for GADA and IAA and 99$^{th}$ percentile for IA-2A. This corresponds to 33.2 WHO units for GADA, 4.3 WHO units for IA-2A and 4.1 WHO units for IAA.

**HLA genotyping**

In Paper II and III, HLA genotyping was done with time-resolved fluorometry based sandwich hybridisation assay (Figure 6), and performed by the research group of Professor Jorma Ilonen in Turku, Finland. The polymerase chain reactions (PCR) were carried out using whole blood or a 3 mm in diameter disc from whole blood spots on filter paper. Specific HLA-DQB1, DQA1 or DRB1 primer pairs, of which one was biotinylated at the 5’-end, were used for amplification of the genes. The PCR product was transferred into a streptavidin-coated microtiter plate together with the detection probe labelled with various lanthanide chelates (Europium, Samarium and Terbium) as described previously.
Subjects & Methods

After an incubation step, allowing the formation of hybrids and to simultaneously collect them onto the wells, the time-resolved fluorescence was detected in a using a Victor™ 1420 Multi label Counter (Wallac Oy, Turku, Finland). Different emission wave length and delay times were used for specific detection of each lanthanide label.

![Diagram](image_url)

**Figure 6** Principle of time-resolved fluorometry. Isolated DNA was amplified with a biotinylated primer. Hybridization was performed with specific DQB1, DQA1 and DRB1 detection probes on streptavidin coated microtiter plates, thereafter time-resolved fluorescence was measured.

**Single nucleotide polymorphism**

In Paper III, CTLA-4, ICOS and PTPN22 SNPs were analyzed by the research group of Professor Jorma Ilonen in Turku, Finland. Three polymorphisms for CTLA-4 were detected as +49 A/G (rs231775), CT60 (+6230) A/G (rs3087243) and CTBC217_1 C/T (rs2033171), one ICOS SNP was detected as CTIC154_1 (c.1624) C/T (rs10932037) and one PTPN22 SNP was detected as +1858 C/T (rs2476601).

DNA was extracted according to a standard salting-out method. Detection of CTLA-4 +49 A/G and PTPN22+1858 C/T SNPs was done after DNA was amplified using primer pairs, of which one was biotinylated at the 5’-end. The biotinylated amplification product was bound to a streptavidin coated microtiter plates and hybridized to one of two lanthanide labelled reporter probe specific
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for the polymorphisms of CTLA-4 +49 A/G or PTPN22 +1858 C/T (Figure 6). Detection was done by time-resolved fluorometry using a Victor™ 1420 Multi label Counter (Wallac).

Detection was done by time-resolved fluorometry using a Victor™ 1420 Multi label Counter (Wallac).

Determination of CT60 A/G (rs3087243), CTBC217_1 C/T (rs2033171) and CTIC154_1 C/T (r10932037) was done using pre-designed primers and TaqMan® probes in real-time PCR based SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). Samples are mixed with two target specific primers and one VIC® and one FAM™ dye-labelled probe, each specific for one SNP where after genotypes are determined.

Preparation of peripheral mononuclear cells

In Paper II, III and IV, PBMC were isolated from heparinized venous blood by Ficoll-Paque™ (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation.

In Paper II, the PBMC were cryopreserved until further analysis. In Paper III, the isolated PBMC were resuspended to 1x10⁶ viable for stimulation, as described below and in Paper IV, PBMC were depleted of the CD25⁺ subset (CD25depleted PBMC) by using anti-CD25-magnetic beads from Dynabeads® before the PBMC and CD25depleted PBMC were used for proliferation assay and stimulation, as described below.

Real-time polymerase chain reaction

Total ribonucleic acid (RNA) was extracted from unstimulated, thawed PBMC (Paper II) or after 72 hours incubation (Paper IV) and converted to cDNA. The mRNA expression was determined by the TaqMan method of real-time PCR, with ribosomal RNA (rRNA) as endogenous control. The comparative Ct
method described by the manufacturer is used to calculate a relative transcription value.

Table 2 All analyzed markers in Paper II and Paper IV, purchased from Applied Biosystems (Foster City, CA, USA).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Paper II</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRNA/18s (Hs99999901_s1)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GATA-3 (Hs00231122_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-4Ra (Hs00166237_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>T-bet (Hs00203436_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-12Rβ1 (Hs00234651_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-12Rβ2 (Hs00155486_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-17A (Hs00174383_m1)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ICOS (Hs00359999_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CTLA-4 (Hs00175480_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Foxp3 (Hs00203958_m1)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IFN-γ (Hs00174143_m1)</td>
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<td></td>
</tr>
<tr>
<td>IL-6 (Hs00174131_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-10 (Hs00174086_m1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IL-2 (Hs00174114_m1)</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

**Stimulation of peripheral mononuclear cells**

In Paper III, the isolated PBMC were resuspended to $1 \times 10^6$ viable cells/mL and were cultured with medium alone, tetanus toxoid (TT), polio virus type 1 /Sabin, CVB4, pertussis toxin or phytohemagglutinin (PHA). After 48 hours, the samples were centrifuged at 400g for 10 minutes and the supernatants were aspirated and stored at -70°C. In Paper IV, the PBMC and CD25depleted PBMC were resuspended to $1 \times 10^6$ viable cells/mL and cultured with medium alone or PHA. After 72 hours, the samples were centrifuged at 400g for 10 minutes and the supernatants were aspirated and stored at -70°C.

**Cell proliferation**

In Paper IV, $1 \times 10^5$ cells/well of PBMC and CD25depleted PBMC were stimulated in a round bottom, 96-well plate with medium alone or in the presence of PHA. The cells were cultured for 6 days and were pulsed with
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[^3]H]thymidine for the last 16-18 hours. The cells were then harvested and assessed for[^3]H]thymidine incorporation in a liquid scintillation counter (1205 Betaplate, Wallac, PerkinElmer Inc, Boston, MA, USA). Results were expressed as a stimulation index (SI=mean counts/min of quadruplicate wells containing cells + antigen divided by mean counts/min of quadruplicate wells containing cells + medium alone). Fifty micro litre cell supernatant was taken from each well before[^3]H]thymidine was added, enabling the measurement of cytokines.

**Enzyme-linked immunsorbent assay**

Cytokine production and C-reactive protein (CRP) were analyzed using enzyme-linked immunosorbent assay (ELISA) (Figure 7). Microtiter plates were coated with monoclonal antibodies against measured proteins and free plastic spaces were blocked with blocking solution. Standards and samples were added to the plates and following incubation, detection was performed by using biotinylated antibodies directed against measured proteins, followed by streptavidin conjugated polyhorseradish peroxidase. Substrate was added and the reaction was stopped by adding 1.8 M sulfuric acid. The responses were expressed in pg/mL and calculated in relation to the standard curve. Those samples that had a value below the limit for quantitative determinations were assigned a value corresponding to half of that value and samples with values above the limit was diluted. Background responses (cultures with medium alone) were subtracted from the response from cultures with antigen induced cytokine concentrations. In Paper III and IV, ELISA was used for analyzing levels of cytokines and in Paper IV, CRP was also measured with ELISA.
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Figure 7 General principle of enzyme-linked immunosorbent assay (ELISA). Cell supernatants are transferred into a microtitre plates coated with antibodies, capturing the protein of interest. A secondary antibody conjugated with biotin is added and thereafter streptavidin bond with an enzyme is. The enzyme reacts with a substrate and form a colored product which can be measured spectrophotometrically. The amount of colored product is proportional to the amount of the specific protein present in the sample.

Multiplex cytokine assay

In paper IV, secreted cytokine levels were determined by commercially available multiplex cytokine assays (Bio-Rad Laboratories Inc, Hercules, CA, USA) on a Luminex 100. Samples or standards were mixed together with a mixture containing an excess of microspheres coupled with antibodies to each cytokine. After incubation and washing, biotinylated detection antibodies were added, followed by streptavidin-phycoerythrin (PE). The fluorescence intensities of the microsphere and detection antibodies were measured. The responses were expressed in pg/mL and calculated in relation to a standard curve. Background responses (cultures with medium alone) were subtracted from the response from cultures with antigen induced cytokine concentrations.
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**Flow cytometry**

Regulatory markers, CD25 and CTLA-4, were analyzed with flow cytometry in Paper IV. Whole blood was incubated with anti-CD4, CD8, CD25 and CTLA-4 antibodies with attached fluorescent dyes. After an incubation step, the erythrocytes were lysed and cells were permeabilized for enabling measurements of intracellular CTLA-4. The cells were analyzed with four-color flow cytometry using a FACSCalibur (BD Bioscience). Lymphocytes were gated from forward- and side scatter and two parameter dot plots were created. CD25<sup>high</sup> was gated according to a slight decrease in CD4 expression. Extracellular and intracellular expression of CTLA-4 and mean fluorescent intensities (MFI) of CTLA-4 was determined in all gated populations.

**Statistical methods**

In Paper I, all data from the questionnaires were optically scanned and manually checked for errors. As a marker for induction of autoantibodies, we used the 90<sup>th</sup> percentile as cut-off and for identification of children at risk of T1D we used the 99<sup>th</sup> percentile, calculated from 5 765 children regarding GADA and 5 635 children regarding IA-2. To identify variables which individually was predictive of the development of autoantibodies, we used a logistic regression model where we used univariate analysis (stage 1). Variables found significant at the level of 0.05 were then included in the multivariate model (stage 2), where all variables were included by stepwise forward selection of the most significant and analyzed simultaneously. Multivariate logistic regression was used to estimate the odds ratio (OR), with a 95% confidence interval (CI), and an OR above 1.0 indicates increased risk.

In Paper II-IV, data were not normally distributed and non-parametric test, corrected for ties, were used. Comparisons between paired groups were analyzed
with Wilcoxon signed-rank test and more than two paired groups with Friedman test. More than two unpaired groups were analyzed with Kruskal-Wallis as a pre-test (correcting for multiple comparisons) with Mann-Whitney U test as post-hoc. For comparison of two unrelated groups, Mann-Whitney U test was used. Correlations were analyzed with Spearman’s rank correlation coefficient test.

Mass significance is a phenomenon due to the fact that statistical analyses are based on probabilities. The probability of finding a difference is 5%, if $p=0.05$ is chosen as significance level. The risk of finding false significances (type 1 error) increases with the number of statistical analyses performed. One way to adjust for multiple tests is to use a lower $p$-value. However, this increases the risk for missing actual differences (type II error). In Paper I, $p<0.05$ was used since we regarded the risk of missing actual differences more problematic than the risk of finding false significances. Differences found were interpreted for whether they were theoretically expected and biologically explainable. In Paper II and IV, no corrections for multiple comparisons were made due to rather few comparisons in each group, and a $p<0.05$ was considered significant. However, in Paper III, adjustments for multiple comparisons were done regarded different HLA-genotypes and the level of significance was lowered to 0.01. For comparisons between three groups, we used Kruskal-Wallis and the method itself considers the multiple groups compared.

All statistical calculations were performed using the statistical package for the social sciences (SPSS) (SPSS Inc., Chicago, Illinois, USA). In Paper I version 11.0 were used, in Paper II version 14 were used, in Paper III version 13 were used and in Paper IV version 15 were used. A $p$-value given as 0.000 by the SPSS program is reported as $p<0.001$. 
Ethical considerations
Type 1 diabetes often develops in children, why studying the environmental and immunological factors influencing the development of T1D among children is important. Research on children should not be performed if similar results may be obtained on adults with their informed consent, only if physiological and pathological states that are particular for childhood will be investigated. This applies to studies of immune and environmental aspects of childhood diabetes. It may seem unethical to include individuals who cannot give informed consent in research projects, but to exclude groups from research can also be unethical and discriminating.

The society benefits from our research if the results lead to better understanding of the factors influencing the development of T1D among children, possibly to eventually enable prediction and prevention of childhood diabetes. The scientific society gains from increased knowledge of important issues regarding general functions of T1D associated factors.

All participating families got written and oral information what it should mean to take part in our studies. They were also informed that the participation was totally voluntary and could be discontinued at any time point without any specific reason. The parents were not automatically informed about genetic risk genotypes or presence of autoantibodies. However, they were told that they could receive this information upon active request, although no effective intervention is available at the moment.

Discomfort during blood sampling was minimized by a topical anaesthetic (EMLA band-aid) and sampling was performed by experienced personnel. All parents /guardians gave their informed consent to enter the studies. Thus, we believe that the ethical benefits exceed the risks in these studies.
All studies were approved by the Research Ethic committee of the Medical Faculty of Linköping University. The ABIS study was also approved by the Research Ethic committee of the Medical Faculty of Lund University.
RESULTS AND DISCUSSION

Several environmental, e.g. virus infections\textsuperscript{134, 136, 140} and early exposure to bovine protein and insulin from cow’s milk\textsuperscript{178}, and immunological risk factors have been associated with the development of T1D. I have hypothesized that some general immunological mechanisms are missing in children that will develop T1D, resulting in an incorrect response to various antigen, enhancing their risk to develop an autoimmune disease like T1D.

In the different projects some aspects of this matter have been studied:

- If environmental factors may induce an autoimmune process in a general population (Paper I).
- If children with increased risk for T1D or with manifest T1D have a different balance between subsets of peripheral blood derived CD4\textsuperscript{+} T cells (Paper II).
- If children in the general population with T1D associated HLA class II risk genotypes or CTLA-4 polymorphisms associated with autoimmune diseases have an altered cytokine response upon T cell stimulation (Paper III).
- If T1D children have a reduced number of CD4\textsuperscript{+}CD25\textsuperscript{high} regulatory T cells or if these cells show a defective function (Paper IV).

Methodological aspects

Autoantibodies

In Paper I, II and III, samples from the ABIS-study were used. Three slightly different cut-off levels were used for the autoantibody measurements. In Paper I, capillary, lysed whole blood was used and as cut-off the 90\textsuperscript{th} and 99\textsuperscript{th} percentile was chosen, both for GADA and IA-2A. These cut-offs identified two different populations of children. The 90\textsuperscript{th} percentile was used to identify children in the general population in whom β-cell autoimmunity has been induced but without
any strong connection to the development of T1D. The 99th percentile was used to identify children with an increased risk of developing T1D, proven as a good cut-off previously115. Insulin autoantibodies were not analyzed due to difficulties measuring IAA in whole blood.

In Paper II and III, we used serum samples and the 98th percentile for GADA and IAA and 99th percentile for IA-2A were used. A high cut-off was chosen due to an increased risk for T1D. We used the 98th percentile instead of the 99th percentile due to decreased sensitivity when threshold was increased, but still with a rather high specificity (Table 3). As very few children had increased levels of IA-2 and the 98th percentile was below the sensitivity level of the method, we used the 99th percentile for IA-2A. Due to differences in values of IA-2A when analyzing whole blood (Paper I) and serum samples (Paper II and III), whole blood generating higher levels, the 90th percentile could be used in Paper I, but only 99th percentile in Paper II.

The cut-off for GADA and IA-2A in Paper I were based on all samples analyzed in our study (5 765 samples for GADA and 5 635 samples for IA-2A) and only the specificity for the 90th and 99th percentile were calculated. The cut-off for GADA and IA-2A in Paper II and Paper III are based on 4007 samples analyzed randomly from the five-year follow-up and the cut-off for IAA is based on 2 481 samples.

Taken part in the Diabetes Autoantibody Standardization Program (DASP) in both 2002 and 2005 gave the specificity and sensitivity shown in Table 3.
Table 3  Specificity and sensitivity from Diabetes Autoantibody Standardization Program (DASP) in 2002 and 2005. n.a.; not analyzed

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>90th</td>
<td>GADA</td>
<td>94%</td>
<td>82%</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>IA-2A</td>
<td>100%</td>
<td>54%</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>98th</td>
<td>GADA</td>
<td>n.a.</td>
<td>n.a.</td>
<td>96%</td>
<td>76%</td>
</tr>
<tr>
<td></td>
<td>IA-2A</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>n.a.</td>
<td>n.a.</td>
<td>99%</td>
<td>30%</td>
</tr>
<tr>
<td>99th</td>
<td>GADA</td>
<td>96%</td>
<td>82%</td>
<td>99%</td>
<td>72%</td>
</tr>
<tr>
<td></td>
<td>IA-2A</td>
<td>100%</td>
<td>55%</td>
<td>100%</td>
<td>72%</td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>n.a.</td>
<td>n.a.</td>
<td>100%</td>
<td>24%</td>
</tr>
</tbody>
</table>

Real-time PCR

In Paper II and IV, data from real-time PCR were included, allowing a fast and sensitive quantification in small samples sizes. However, discrepancies may exist between the expression of mRNA and protein levels. In general, several methods can be used to evaluate the expression of a specific marker, for example expression of specific mRNA, secretion of the protein or expression of the protein in cells, with flow cytometry.

Real-time PCR was chosen for Paper II due to several reasons. Only small volumes of blood from the children were received due to ethical and practical reasons, limiting the methods which can be used. In addition, as expression of various markers was studied in unstimulated cells, where cytokine secretion is low, we did not measure the secreted cytokines. Also, most cytokines are not detectable or close to the detection limits in plasma/serum. Although Western Blot can be used for analyzing proteins, this did unfortunately not work properly...
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in unstimulated cells. Also for Paper IV, we chose to measure mRNA expression
as we were interested in the spontaneous expression of cytokines.

In both Paper II and Paper IV the comparative Ct method was used. In each
quantitative PCR method, differences in starting material will introduce errors.
Therefore normalization by a house-keeping gene is required to correct for these
experimental variations. In both Paper II and Paper IV, rRNA was used for this
purpose. Phytohemagglutinin stimulated PBMC from four healthy donors, from
whom cDNA has been pooled, was used as an in-house calibrator, and all
samples were related to this.

The comparative Ct method uses arithmetic formulas to achieve a relative
quantification and the amount of target gene, normalized to an endogenous
reference (rRNA, 18s) and relative to a calibrator, is given by the formula
$2^{-\Delta \Delta Ct}$ (User Bulletin #2, Applied Biosystems). For each calculation to be valid,
the efficiency of the target amplification and the efficiency of the endogenous
reference must be approximately equal. This has been tested by looking at how
$\Delta Ct$ varies with template dilution. Dilutions ranged from 1 to 1/512 and after the
run, $\Delta Ct$ was plotted against log input amount. The slope should be <0.1 for
each measured target gene. In Figure 8, ICOS is shown as an example and all
target genes, from both Paper II and IV, passes this test.
Figure 8  Plot over Ct values against log input amount for ICOS. The dilution ranged from 1 to 1/512 and the absolute value of the slope should be close to 0, but in any case <0.1. \( \Delta \text{Ct} \) of ICOS showed a slope of -0.04, which passes the test.

**Optimization of antigen stimulations**

In Paper III, stimulation of PBMC with antigens was optimized using PBMC collected from children not participating in the study. The stimulation was performed as described in Paper III. Different incubation times (24, 48 and 72 hours) were tested and 48 hours were found to be optimal. Different concentrations were tested for each antigen (TT: 5, 10 and 20 Lf/mL; Polio: 0.1,0.5 and 1.0 µg/mL; CVB4: 0.1, 0.5, 1.0 µg/mL; Pertussis: 0.5, 1.0, 2.0 µg/mL) and the optimal concentrations were found to be 20 Lf/mL of TT, 0.5 µg/mL of Polio, 0.5 µg/mL of CVB4, 2 µg/mL Pertussis. Optimal concentrations and incubation times of PHA and anti-IL-4R have been optimized previously.

**Gating strategy in flow cytometry analysis**

The methods of gating regulatory T cells have been discussed carefully. As the CD4\(^+\)CD25\(^+\) phenotype is displayed by activated T cells, the ability to identify regulatory T cells with this marker is limited. Several efforts have been made to
combine different surface markers for a more specific characterization and commercially available antibodies against Foxp3 can further support the identification of regulatory T cells. However, at the time of sample collection we had no possibility to study the expression of Foxp3 or CD127 due to lack of suitable antibodies. Therefore lymphocytes were identified by their forward- and side- scatter properties (Figure 9a) and the CD4^+^CD25^{high} population was gated according to the highest expression of CD25 and a slight decrease in CD4 intensity after regions were set individually for each child with corresponding isotype control (Figure 9b).

![Representative flow cytometric plots from a T1D patient showing gating strategy](image)

**Figure 9** Representative flow cytometric plots from a T1D patient showing gating strategy used to define CD4^+^CD25^{high} T cells. Lymphocytes were gated according to their forward- and side-scatter properties (a) and examined for expression of CD4 and CD25. CD4^+^CD25^{high} T cells were defined as lymphocytes positive for CD4 staining with the highest expression of CD25 together with a slight decrease in CD4 expression (b).

For intensity of intra- and extra cellular CTLA-4, MFI were calculated after correction for instrument variation by QC3™ microbeads, allowing CV of 15% for each fluorescent dye.

**Magnetic depletion**

In Paper IV, we used magnetic sorting for the depletion of CD4^+^CD25^+^ T cells. The optimal way to select a specific population would be by cell sorting on a
flow cytometer, but since we had no cell sorter available we chose magnetic depletion for the depletion of CD25$^+$ cells. This is a rather cheap method, needing nothing else than you sample, magnetic beads and a magnet. However, magnetic cell sorting don’t always allow for a stringent isolation of cells. The intention in Paper IV was to remove the CD4$^+$CD25$^+$ regulatory T cells and we examined the result with flow cytometry, individually for each sample, making sure that the population of regulatory cells were depleted after treatment. (Figure 10)

![Figure 10](image.png)

**Figure 10** Representative flow cytometric plots from a child showing depletion of CD4$^+$CD25$^+$ T cells. Lymphocytes were examined for expression of CD4 and CD25. CD4$^+$CD25$^{\text{high}}$ T cells were defined as lymphocytes positive for CD4 staining with the highest expression of CD25 together with a slight decrease in CD4 expression (a). After magnetic depletion the CD4$^+$CD25$^{\text{high}}$ T cells was absent in the sample, verifying a successful depletion (b).

**Influence of environment**

In studies investigating environmental factors, an ordinary set up is using children with increased genetic risk, who are followed with respect to autoantibody development and development to clinical manifest T1D. Thus we investigated whether environmental factors could trigger signs of β-cell autoimmunity during the first year of life in an unselected population of children (Paper I). Out of 5 765 samples analyzed for GADA and 5 635 samples analyzed for IA-2A, 582 had levels of GADA above the 90th percentile and
Results & discussion

57 above the 99th percentile. For IA-2A, the corresponding figures are 574 at the 90th and 57 at the 99th percentile. 126 samples had both GADA and IA-2A above the 90th percentile and 6 samples above the 99th percentile. The appearance of autoantibodies in young children is a dynamic process and some of the autoantibody positive children will revert back to negativity184. As this fluctuation is more likely to appear among children with lower autoantibody level than the 99th percentile, the 99th percentile was used as marker for increased risk of T1D. Children with high levels of two or more autoantibodies have the highest risk of developing clinical T1D185 and in Paper I, six children had multiple antibodies above 99th percentile at one year of age. However, at later time points all children had reverted back to autoantibody negativity and none of them had in November 2007 converted to clinical T1D.

The risk for developing autoantibodies above 90th percentile was associated with several factors analyzed (Table 4).

**Table 4** Risk factors related with increased levels of β-cell autoantibodies to IA-2A and GADA, above the 90th percentile, in an unselected population. The odds ratio (OR), confidence interval (CI) and p-values from a stepwise logistic regression analysis are shown.

<table>
<thead>
<tr>
<th>Risk factors &gt;90th percentile</th>
<th>IA-2A [OR (95% CI)]</th>
<th>GADA [OR (95% CI)]</th>
<th>IA-2A and GADA [OR (95% CI)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys/Girls</td>
<td>1.3 (1.1-1.6)a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celiac disease among grandparents</td>
<td>2.2 (1.1-4.4)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>1.0 (ref)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>1.3 (1.0-1.8)ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>1.5 (1.1-2.0)c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>0.9 (0.7-1.3)ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal gastroenteritis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Month 7 during pregnancy</td>
<td>1.1 (1.0-1.3)d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/medium vs high</td>
<td>1.5 (1.2-1.9)e</td>
<td>1.4 (1.1-1.7)f</td>
<td></td>
</tr>
</tbody>
</table>

a p=0.007, b p=0.027, c p=0.004, d p=0.028, e p=0.001, f p=0.003
This suggests that environmental factors might contribute to the development of β-cell autoimmunity. Interestingly, these factors were the same which previously have been associated with the development of T1D in several studies. Increased risk of IA-2A above 90th percentile in children born during spring may suggest that environmental factors very early in life \(^{150}\), or even during pregnancy \(^{134, 135}\), may play a role in the development of β-cell autoimmunity. The finding of gastroenteritis during pregnancy as a risk factor for both GADA and IA-2A also supported the theory of maternal influences of the children’s immune system during the perinatal period.

Children with levels of GADA and/or IA-2A above the 99th percentile more often had a first-degree relative with T1D, OR 2.6 (95% CI 1.0-6.6; p=0.042). The risk of developing T1D has previously been associated with T1D in first degree relatives \(^{131}\). Offspring of mothers with T1D have a risk of approximately one to five percent of developing T1D, whereas the risk is about five to eight percent if the father is affected \(^{186-188}\). In brothers or sisters the risk varies between six and 20%, depending on their degree of HLA identity \(^{131-133}\). As we investigated an unselected population of children at one year of age, we did not have enough cases for separate analysis of the effect of diabetes in the parents or siblings. It would however be interesting to investigate this relation in the ABIS material now, when the children are nine to eleven years old, or in the future when they are even older, with both β-cell autoimmunity and T1D as primary outcomes.

The presence of GADA above the 99th percentile was associated with grandparents having T2D, OR 2.1 (95% CI 1.1-4.3; p=0.033) and the prevalence of T1D and T2D has also previously been shown to occur rather often in the same families \(^{131, 189, 190}\). We have in follow-up analysis confirmed the association between T2D in first-degree relatives and the occurrence of
autoantibodies\textsuperscript{153}. This suggests that the etiological factors of T1D and T2D may, at least partly, be shared, which fits with the β-cell stress hypothesis\textsuperscript{148}.

Early exposure to cow’s milk (during the first two months in life) was also associated with high levels of autoantibodies, especially IA-2A, OR 2.9 (95% CI 1.5-5.5; p=0.001). Early introduction of cow’s milk based formula, together with high consumption of fresh cow’s milk at one year of age, has also been associated with higher β-cell autoantibodies in older children\textsuperscript{153}. Associations between cow’s milk, T1D and induction of IA-2A have previously been shown\textsuperscript{151}, even though contradicting results exist\textsuperscript{152}. As the importance of cow’s milk in the induction of β-cell autoantibodies and T1D is debated, a large double-blinded prospective intervention study have been started, aiming to determine the relationship between early introduction of cow’s milk and the development of T1D\textsuperscript{191}.

Our results, together with others, emphasize the complexity around environmental factors and the importance of these factors at an early stage in the process against β-cell autoimmunity. However, as most children with autoantibodies do not develop T1D, other factors likely modify the effect of the environmental triggers and may provide protection against T1D. One of these factors may be the HLA class II protective haplotypes. When we studied the effect of protective haplotypes on response to enterovirus stimulation (Paper III) we found that the DQB1*0602 (DR2-DQ6) allele was associated with a higher IFN-γ and IL-2 response to polio virus and the DQA1*05-DQB1*0301 (DR5-DQ7) allele with higher IL-6 and IL-2 response to CVB4 (Figure 12a-d). This might indicate a stronger immune response against enterovirus and contribute to protection from enterovirus infections\textsuperscript{192}.
Effect of genetic risk factors on immune responses

Human leukocyte antigen class II region on chromosome 6p21 encodes the most important genetic factor in T1D and previous studies have reported aberrant cytokine responses in association with HLA class II risk haplotypes\(^ {90-92}\), however multiple non-HLA genes also contribute to disease development. The gene encoding CTLA-4 on chromosome 2q33 \(^ {80,95}\), ICOS mapped to the same chromosome, 2q33, as CTLA-4 and recently LYP, encoded by the PTPN22 gene on chromosome 1p13, have all been implicated in autoimmune diseases like T1D \(^ {81,82}\). All of these genes are actively involved in the function of the immune system, in regulating the activation of T cells. The HLA DQ and DR alleles influence T1D susceptibility \(^ {85,86}\) and among Caucasians, DRA1*0401/2/4/5-DQB1*0302, encoding DR4-DQ8, and DQA1*0501-DQB1*0201, encoding DR3-DQ2, have been suggested to have the strongest association with T1D.

Studies of the HLA-peptide binding domain demonstrate that different HLA alleles bind different epitopes, maybe even from the same antigen \(^ {193}\) and susceptibility and protective HLA class II molecules could bind and present non-overlapping peptides \(^ {194}\).

The profile of T\(_H\) cells in peripheral blood derived unstimulated lymphocytes was in Paper II investigated in children with HLA class II haplotypes associated with T1D. In Paper III, children with T1D associated HLA class II haplotypes, CTLA-4 polymorphisms, ICOS polymorphism or PTPN22 polymorphism associated with autoimmune diseases were investigated with respect to cytokine responses upon T cell stimulation. No remarkable up- or down regulation of markers for different T\(_H\) subtypes was seen in children with the risk haplotypes DQA1*05-DQB1*02 (DR3-DQ2) and DRB1*0401/2/4/5-DQB1*0302 (DR4-DQ8), either with risk haplotypes in combination with each other or with neutral haplotypes (Figure 11). This indicates that HLA risk haplotypes do not have an impact on a general polarization of T\(_H\) cells in peripheral blood.
Figure 11 The mRNA expression of various immune factors was similar in children with HLA risk haplotypes DQA1*05-DQB1*02 (DR3-DQ2) and DRB1*0401/2/4/5-DQB1*0302 (DR4-DQ8) (filled squares) and in healthy children without any risk for T1D (open squares). Horizontal lines indicate median values.

In Paper III, cytokine responses to different antigens were investigated with respect to different genetic risk factors associated with T1D. As our group previously explored the relationship between HLA and CTLA-4 +49 SNP risk factors and cytokine response to T1D related antigens, the aim in Paper III was to study whether additional risk genotypes related to T1D were associated with an aberrant cytokine response in general.

As most Swedish children follow the recommended vaccination program and are thus vaccinated against TT, pertussis toxin and polio virus, almost 100% of the children respond with cytokine secretion to these antigens. Also enterovirus
infections are common and responses to CVB4 are thus seen in many of the children. Children with the risk haplotype DQA1*05-DQB1*02 (DR3-DQ2) had a higher IL-13 response to TT stimulation (p=0.002, Paper III). Different HLA alleles have the ability to present different peptides in different ways. The elevated IL-13 response seen among children with DQA1*05-DQB1*02 (DR3-DQ2) might be a result of this. As IL-13 can be regarded as a marker of Th2 response this might also suggest an impaired polarization against a Th1 immune response in response to TT. This kind of impaired Th1 response has earlier been shown among T1D patients. As no other differences were seen in children with the two different HLA risk haplotypes DQA1*05-DQB1*02 (DR3-DQ2) or DRB1*0401/2/4/5-DQB1*0302 (DR4-DQ8), it seems that children with T1D associated risk haplotypes do not have disposition for developing aberrant cytokine responses in general. These results are in accordance with the previous finding of no general T cell polarization among unstimulated peripheral blood T cells (Paper II).

Children with the protective haplotype DQB1*0602 (DR2-DQ6) had higher IFN-γ (p<0.001) and IL-2 (p=0.005) in response to polio stimulation, compared to other genotypes (Figure 12a-b). Polio virus belongs to the family of enteroviruses, which have been associated with T1D and the seroconversion to β-cell autoantibody positivity. Interferons have the ability to induce an antiviral response in human pancreatic islet cells, especially true for IFN-α, but also IFN-γ showed the same tendencies. Our results may support this view and indicate that the response seen in children with the haplotype DQB1*0602 (DR2-DQ6) could contribute to a better protection from enterovirus infections and minimize the risk of accelerating β-cell destruction. Stimulation with CVB4 did not however show the same increased IFN-γ response in children with DQB1*0602 (DR2-DQ6). Instead, the eleven children
with the protective haplotype DQA1*05-DQB1*0301 (DR5-DQ7) showed a higher IL-6 (p=0.039) and IL-2 (p=0.015) response against CVB4 than the children with other genotypes (Figure 12c-d).

**Figure 12** The protective HLA haplotypes DQB1*0602 (DR2-DQ6) was associated with higher production of IFN-γ (a) and IL-2 (b) in response to polio stimulation while children with the protective haplotype DQA1*05-DQB1*0301 (DR5-DQ7) was related to increased production of IL-6 (c) and IL-2 (d) in response to coxsackie virus (CVB4). Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown. Note the different scales for the y-axis.
The haplotype DQB1*0501 (DR1-DQ5), which is considered as a neutral haplotype in regard to T1D risk, was associated with increased secretion of IL-10 in response to polio stimulation (p=0.003) and a higher secretion of IL-6 in response to TT (p=0.001) (Paper III).

The connection between CTLA-4 +49 A/G polymorphism and autoimmune diseases has been investigated and show a greater proliferative response of T cells from individuals with +49 G/G genotype and lower expression of CTLA-4 in CD4+CD25high T cells among children with +49 G/G genotype, as well as an increased expression in +49 A/A individuals. The CTLA-4 is also involved in reducing the contact period between HLA and TCR molecules and a decreased expression of CTLA-4 on cell surfaces may increase this contact and induce T cell activation and cytokine release. CT60 G/G has also been associated with a lower expression of soluble CTLA-4, although discrepant results exist. We investigated whether these polymorphisms, together with CTBC217_1 C/T, were associated with an aberrant cytokine response, as CTLA-4 inhibits cytokine production in activated T cells. Usually transcription factors such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and NFAT cause an up-regulation of the cytokines IL-2, IFN-γ and IL-4 upon T cell activation. This signal is inhibited by CTLA-4 mediating a down-regulated cytokine production. Thus, a decreased expression of CTLA-4 due to polymorphisms could lead to increased T cell activation and higher secretion of cytokines, which could increase the risk for autoimmune diseases, like T1D.
Stimulation of lymphocytes with bacterial toxins and viruses resulted in a higher IFN-γ secretion in children with the CTLA-4 genotypes associated with T1D risk. For instance, pertussis stimulation induced a higher IFN-γ secretion in children with the +49 G/G SNP (p=0.034, Kruskal-Wallis test) (Figure 13a) and CTBC217_1 T/T (p=0.015, Kruskal-Wallis test) (Figure 13b).

Also, after PHA stimulation, children with CTBC217_1 T/T had a higher IFN-γ secretion (p=0.046, Kruskal-Wallis test) (Figure 14).
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Figure 14 Children with the CTBC217_1 T allele showed higher IFN-γ production in response to Phytohemagglutinin (PHA) stimulation. Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown.

Similar tendencies were observed for the genotype CT60 G/G, with higher IFN-γ production both after PT and PHA stimulation (p=0.055 and p=0.062, respectively, Kruskal-Wallis test) (Figure 15a-b).
Figure 15 Stimulation with pertussis toxin (PT) (a) and phytohemagglutinin (PHA) (b) tended to induced a higher IFN-γ production in children with CT60 G/G haplotype. Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown. Note the different scales for the y-axis.

The increased IFN-γ production was seen as a response to mitogen and antigen specific stimuli, confirming previous results by our group and fitting with the view of CTLA-4 down-regulating immune response in general and not as an antigen-specific mechanism. This is in accordance with the idea of more activated T cells in association with CTLA-4 polymorphisms. However, the secretion of IL-2 or IL-4 was not affected by any CTLA-4 polymorphism, suggesting that the CTLA-4 SNPs investigated might regulate T cell inactivation through other, more general, mechanisms than though AP-1, NF-κB and NFAT, for instance by the disassociation of cSMAC or in reducing the contact period between HLA and TCR molecules. T helper 2 cells, prominent IL-4 producers, have higher levels of CTLA-4 compared to TH1 cells and
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decreased expression of CTLA-4 may thereby not influence the IL-4 production as much as the IFN-\(\gamma\) production.

How may increased IFN-\(\gamma\) response be both beneficial and harmful, as children with the HLA class II protective haplotypes DQB1*0602 (DR2-DQ6) and DQA1*05-DQB1*0301 (DR5-DQ7) and also children with CTLA-4 SNP associated with T1D risk were found to have higher IFN-\(\gamma\) secretion (Paper III)? Enteroviruses have for a long time been associated with the development of T1D and children with HLA class II risk haplotypes have been found to have a decreased IFN-\(\gamma\) response to CVB4. Our result, with an increased cytokine response in children with protective haplotypes, indicates a possible contribution to protection from enteroviruses. However, the high IFN-\(\gamma\) response with T1D associated CTLA-4 alleles may contribute to a higher general activation of the immune system and thus be associated with T1D pathogenesis.

The possible associations between SNPs in the ICOS gene and autoimmune diseases made us investigate the CTIC154_1 C/T SNP.

Fiftyfive children (83.5%) in Paper III had the C/C genotype, nine (14.5%) carried the C/T genotype and only two children (3%) had the T/T genotype. Children with the T/T genotype did show a lower IL-6 response to PHA and a higher IL-13 response to pertussis stimulation. This is interesting since polymorphisms in the ICOS gene have been associated with Th2 cytokine production. However, any clear conclusion could not be made since there were only two individuals with T/T genotype.

Lymphoid Tyrosine Phosphatase is a protein strongly associated with negative regulator of T cell activation and the SNP at position +1858 in the gene PTPN22, encoding LYP, is associated with T1D. The +1858T allele
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gives a gain of function, which means that individuals with this allelic variant would have an increased inhibition and a lower T cell response to antigenic stimulation \(^\text{107}\). In Paper III, 85.5% of the children had the C/C genotype and 14.5% had the T/C genotype. The T/T genotype is very rare and none of the children carried this genotype.

The stimulated cytokine responses were similar in children with the C/C genotype and the T/C genotype, although another study has shown reduced IL-10 secretion in heterozygous individuals \(^\text{108}\). Unfortunately, since none of the children had the T/T genotype we could not investigate the full effect of this SNP on cytokine secretion.

**Immunological balance**

T helper cells and regulatory T cells are important players in the immune reactions and unbalance between these cellular subtypes could contribute to the development of autoimmune reactions leading to a chronic autoimmune disease, such as T1D. Paper II and IV discuss this topic through the examination of general activity of T\(_\text{H}1\), T\(_\text{H}2\), T\(_\text{H}17\) and regulatory T cells, as well as the function of regulatory T cells.

Children with an autoimmune process showed a decreased expression of GATA-3 in unstimulated PBMC compared to healthy children without any risk factors associated with T1D (p=0.014) and children with HLA risk haplotypes alone (p=0.032), but the children with genetic risk alone did not differ from the control children without genetic risk (Figure16).
The Relative transcription of GATA-3 was lower in children with autoantibodies (Aab+) above 98th percentile for GADA and IAA or 99th percentile for IA-2A compared to children without increased risk for T1D (Control) and children with HLA risk genotypes (HLA risk). Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown.

Inhibition of GATA-3 seems to be more important than increased levels of T-bet for the production of IFN-γ, and thus the low levels of GATA-3 could support the development of Th1 immune response capable for destruction of β-cells. No differences in expression levels of IL-4Rα, T-bet or IL-12R were observed, however (Table 6).
Table 5 The relative transcription of markers associated with TH1 and TH2 in children without increased risk for T1D (control), children with HLA risk genotypes associated with T1D (HLA risk) and children with autoantibodies above 98th for GADA and IAA or 99th percentile for IA-2A (aab+).

<table>
<thead>
<tr>
<th></th>
<th>Control median (range)</th>
<th>HLA risk median (range)</th>
<th>Aab+ median (range)</th>
<th>p-value (Kruskal-Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4Rα</td>
<td>17.5 (9.0-31.7)</td>
<td>15.5 (9.6-39.1)</td>
<td>18.3 (7.3-44.4)</td>
<td>0.73</td>
</tr>
<tr>
<td>T-bet</td>
<td>5.4 (1.9-19.7)</td>
<td>7.1 (0.5-17.9)</td>
<td>2.9 (1.3-10.7)</td>
<td>0.24</td>
</tr>
<tr>
<td>IL-12Rβ1</td>
<td>15.8 (2.7-51.5)</td>
<td>17.1 (2.1-34.2)</td>
<td>15.8 (5.1-27.3)</td>
<td>0.86</td>
</tr>
<tr>
<td>IL-12Rβ2</td>
<td>3.9 (0.5-21.7)</td>
<td>2.9 (1.2-6.9)</td>
<td>3.0 (0.5-32.1)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

In contrast to children at risk of T1D, diabetic children showed lower levels of IL-12Rβ1 (Figure 17a) and IL-4Rα (Figure 17b) mRNA at the diagnosis of the disease, as well as in samples taken after three and 12 month duration, when compared to the healthy control children.

Figure 17 The relative transcription of IL-12Rβ1 (a) and IL-4Rα (b) was lower in T1D children, at all follow-up times, compared to healthy control children (control). Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown. Note the different scales for the y-axis.
The expression of T-bet was also significantly lower at the disease diagnosis compared to controls with similar tendencies in later samples (Figure 18). This decreased expression of several T cell polarization markers may reflect a general exhaustion of the immune system at the time of clinical manifestation of T1D. The decreased expression of IL-4Rα, T-bet and IL-12Rβ1 also supports previous findings of decreased expression of IL-4 and IFN-γ in PBMC 171.

**Figure 18** The relative transcription of T-bet was lower in T1D children ten days after diagnose compared to healthy control children (control). Later time points showed the same tendencies, although not significant. Horizontal lines indicate median values and p-values of Mann-Whitney U-test are shown.

T helper 17 cells were recently discovered to be a subset of T<sub>H</sub> cells separate from T<sub>H</sub>1 and T<sub>H</sub>2 cells. These T<sub>H</sub>17 cells have been associated with several
autoimmune disorders\textsuperscript{206-210} and they produce the pro-inflammatory cytokines IL-17A, IL-17F and IL-22\textsuperscript{38,39}. T\textsubscript{H}17 cells may also be important in the development of T1D although the association with T1D have not been confirmed yet. In unstimulated peripheral PBMC, the transcription of IL-17A was low in general and there were no differences between healthy children, children at risk or T1D children (data not shown).

Regulatory T cells are important in down-regulation of the immune responses and controlling the autoreactive T cells present peripherally\textsuperscript{211}. Regulatory T cells have been linked with the expression of CD25\textsuperscript{48}, CTLA-4\textsuperscript{50,51}, Foxp3\textsuperscript{55,56} and ICOS\textsuperscript{53} and the regulatory capacity of CD4\textsuperscript{+}CD25\textsuperscript{high} regulatory T cells has been investigated in several different autoimmune diseases\textsuperscript{172,212-216} as well as in allergy\textsuperscript{217,218}.

We did not have the opportunity to do functional studies of regulatory T cells in children with increased risk of T1D. However, the PBMC from children with risk of T1D showed similar expression levels of the markers Foxp3, CTLA-4 and ICOS as was seen in healthy control children (Figure 19), suggesting a comparable number of regulatory T cells in pre-diabetic children. Also in T1D children, the expression of CTLA-4 and ICOS was similar as in healthy control children (data not shown).
Figure 19 The relative transcription of markers associated with regulatory T cells did not differ between children without any risk (open squares), children with HLA risk genotype (filled squares) and children with autoantibodies above 98th percentile for GADA and IAA or 99th percentile for IA-2A (filled triangles). Horizontal lines indicate median values and p-values of Kruskal-Wallis test are shown.

Foxp3 showed similar levels of expression in T1D children and in healthy control children ten days after diagnosis (Paper II and IV) and three months after diagnosis (Paper II). However, Foxp3 expression was decreased 12 months after diagnosis of T1D when compared to healthy children (p=0.027, Paper II). The Foxp3 expression was lower also in diabetic children 12 months after diagnosis.
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when compared to the samples taken at diagnosis and at three months (p=0.038 and p=0.056 respectively, Paper II).

As the expression of Foxp3, CTLA-4 and ICOS has been shown to increase as a response to insulin treatment in newly diagnosed T1D patients, this suggests that the diabetic children have an activation of insulin-specific regulatory T cells at diagnosis. This may be a reason why no differences were seen in these markers between T1D children and control children at diagnosis; the insulin treatment may correct the impaired regulatory function. This might also be a reason of the decreased expression of IL-4Rα, T-bet and IL-12Rβ1 seen in Paper II, since the regulatory compartment might be effective in diminishing the immune response.

Investigating the proportion of CD4+CD25^{high} T cells with flow cytometry during 18 months follow-up after T1D diagnosis revealed no differences between diabetic and healthy children at any time point (Paper IV) and the amount of CD4+CD25^{high} T cell expressing extracellular or intracellular CTLA-4 was similar in both groups. However, the diabetic children had more intracellular CTLA-4 per CD4+CD25^{high} T cell represented as the MFI of intracellular CTLA-4, than the healthy children. This was seen at ten days after diagnosis (Figure 20) and may reflect an ongoing activation of regulatory T cells during T1D debut. No significant differences were observed in the expression level of intracellular CTLA-4 during the follow-up among T1D children, however. A general activation of the immune system was also seen as increased levels of serum CRP at ten days and three months after diagnosis (Paper IV). C-reactive protein is induced by IL-6, which is secreted in great quantity from the innate immune system, and activates macrophages and the complement system. The increased levels of CRP might reflect the inflammatory process taking place in pancreas.
Figure 20 The T1D children had higher expression of intracellular CTLA-4 per CD4^{+}CD25^{high} T cell than the healthy control children ten days after diagnosis. The horizontal line indicates median value and p-values of Mann-Whitney U-test are shown.

Interestingly, a negative correlation between insulin dose and the level of Foxp3 was seen at 3 months after diagnosis (R=-0.541; p=0.030, Paper II). This could support the activation of insulin-specific regulatory T cells and indicates that activation of regulatory T cells near diagnosis may control the β-cell destruction and thus be related to the insulin dose needed. The activation of T effector cells increase the Foxp3 expression^{62-64}, trying to diminish the immune activation^{65}. The loss of Foxp3 expression from diagnosis toward 12 month duration (Paper II) might be a result of diminishing regulatory activity during the fading autoimmune attack with final loss of β-cells.

A negative correlation of IFN-γ expression with the production of C-peptide was observed ten days after diagnosis (r=-0.676, p= 0.004, Paper IV), suggesting that a more pronounced inflammation may lead to a more rapid loss of β-cells and insulin production.
The effect of regulatory T cells on effector cells was investigated using depletion of CD4⁺CD25<sup>high</sup> T cells. Real-time PCR showed that expression of Foxp3 was significantly higher in PBMC than in CD25-depleted PBMC (Paper IV), which is reasonable due to the expression of Foxp3 in CD4⁺CD25<sup>high</sup> cells. We also saw a higher expression IL-10 in PBMC compared to CD25-depleted PBMC (Paper IV) and the spontaneous secretion of TGF-β in PBMC was also higher than in CD25-depleted PBMC (Paper IV), although this difference could not be seen at mRNA level. Transforming growth factor-β and IL-10 are cytokines known to be involved in regulation of immune response and TGF-β has been shown to up-regulate the expression of Foxp3 for the induction iTreg<sup>222</sup> and maintaining suppressor function in nTreg cells<sup>59</sup>. Interleukin-10 has been shown to be important for the induction of the Tr1 cells<sup>223</sup>, the regulatory cells which mediate their suppression by local secretion of IL-10 and TGF-β<sup>224</sup>. A higher production of TGF-β and IL-10 in PBMC than in CD25-depleted PBMC suggests that these cytokines are produced by CD4⁺CD25<sup>high</sup> T cells<sup>213,225</sup>. The results verify that the depletion of regulatory T cells worked using our method and since the same phenomenon was seen both among T1D and healthy children the results suggest a normal functional activity of CD4⁺CD25<sup>high</sup> regulatory T cells in T1D<sup>220</sup>.

Stimulating PBMC and CD25-depleted PBMC with PHA resulted in a more pronounced proliferative response among CD25-depleted PBMC than in whole PBMC in diabetic children (median SI 25.2 and 13.6, respectively, p= 0.009). Also the spontaneous expression of IFN-γ was higher in the CD25-depleted PBMC as compared to whole PBMC population (Paper IV) in the diabetic children at ten days after diagnosis. Accordingly, the presence of CD4⁺CD25<sup>high</sup> regulatory T cells in PBMC population inhibited proliferation response of the cells and expression of IFN-γ. Although we did not find any significant
difference in the proliferation response of CD25depleted PBMC and PBMC in the healthy children this might be due to rather small numbers of healthy children studied since the median SI was somewhat higher in CD25depleted PBMC compared to PBMC in healthy children (median SI 22.4 and 20.5, respectively). We did not find any difference in SI between diabetic and healthy children (data not shown), supporting a normal function of the regulatory T cells among T1D children.
SUMMARY AND CONCLUDING REMARKS

In this thesis the association between environmental factors and β-cell autoimmunity was evaluated, as well as the relationship between possible immunological aberrancies and increased risk for T1D in children was elucidated. Different environmental factors, as well as immune dysregulation have been proposed to interact, triggering an autoimmune response leading to overt T1D. The mechanism behind this is still largely unknown and maybe some general immunological mechanisms are missing in children that will develop T1D, making them more susceptible for the disease.

Several environmental factors such as early nutritional factors and early infections may induce islet autoantibodies in a general population, but the mechanisms of the induction are still elusive. It should be emphasised that the induction of autoantibodies, especially single autoantibodies, may not always lead to the development of manifest T1D and other factors, protective or triggering, have to be involved as well. The results in this thesis further showed that responses to antigens was dependent on HLA genotypes presenting antigens and that down regulation of immune response was controlled in part of CTLA-4. The T1D associated HLA haplotypes and different CTLA-4 polymorphisms may thus be associated with T1D pathogenesis.

The findings do not support a systemic activation of the immune system in children at risk of T1D. Instead there is a decrease in T_{H1} and T_{H2} markers after T1D manifestation, possibly as a result of activated regulatory mechanisms trying to dampen the autoimmune reaction taking place. The studies performed did not reveal impaired function of the peripheral blood derived regulatory T cells in T1D, but rather activated and functional compartment of CD4^{+}CD25^{high} regulatory T cells, supporting the theory of regulatory T cells suppressing an ongoing inflammation.
In summary, several genetic and environmental factors seem to contribute to T1D and this thesis emphasizes the importance of multiple mechanisms acting in concert. The results raise the question whether the immunological enigma of T1D could be revealed by studies of peripheral blood.
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****************************************************************

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