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# Immunological profile and aspects of immunotherapy in type I diabetes

Maria Hjorth

Division of Pediatrics

Department of Clinical and Experimental Medicine

Faculty of Health Sciences, Linköping University

SE-581 85 Linköping, Sweden



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During the course of the research underlying this thesis, Maria Hjorth (formerly Hedman) was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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*To my family*



## Abstract

Type 1 diabetes (T1D) is a chronic, autoimmune disease caused by a T cell mediated attack on the insulin producing pancreatic  $\beta$ -cells. Even though reasonable quality of life can be acquired with modern insulin therapy, prevention of acute and late serious complications is facilitated by preservation of residual insulin secretion. Preventing  $\beta$ -cell destruction is therefore an important goal of T1D therapy. Characterisation of immunological changes in the course of T1D is essential for understanding the underlying pathogenic mechanisms and for evaluating the effect of therapeutic intervention.

This thesis aimed to study the immune profile in individuals at increased risk of T1D and in patients diagnosed with the disease. In addition, the immunological effects of treatment with the B vitamin, Nicotinamide, and by antigen-specific immunotherapy using GAD<sub>65</sub>, have been studied in high-risk individuals and in T1D patients.

We have found that individuals at high risk of T1D had an increased T helper (Th) 1 like immune profile, defined by high secretion of interferon (IFN)- $\gamma$ . At the time of clinical onset of T1D, the Th1 dominance was diminished. Children with newly diagnosed T1D had a suppressed Th1 like profile, detected by chemokine and chemokine receptor profile. This was accompanied by an induced population of CCR7<sup>+</sup> and CD45RA<sup>+</sup> naïve, CD8<sup>+</sup> cytotoxic T (Tc) cells and a reduced CD45RO<sup>+</sup> memory Tc cell pool.

Oral treatment with Nicotinamide has been shown to be ineffective in preventing T1D. However, we have found a decreased secretion of IFN- $\gamma$  in high-risk individuals receiving the treatment. We have previously shown that subcutaneous injections with GAD-alum in T1D children induced a better preservation of endogenous insulin secretion compared with placebo. Here, we demonstrate that the treatment induced an early GAD<sub>65</sub>-specific Th2 and regulatory immune profile. After a few months, and still after more than two years, the recall response to GAD<sub>65</sub> was characterised by a broader range of cytokines. GAD-alum treatment also induced a GAD<sub>65</sub>-specific CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cell population and reduced the levels of CD4<sup>+</sup>CD25<sup>+</sup> cells.

In conclusion, a Th1 like immune profile in pre-diabetic individuals indicates an imbalance of the immune system. At time of clinical onset, and in the period afterwards, reduction of the Th1 associated immune response could be an effect of a suppressed destructive process, selective recruitment of effector T cells to the pancreas or a defective immune regulation. The protective effect of GAD-alum in T1D children seems to be mediated by an early skewing of GAD<sub>65</sub>-induced responses towards a Th2 phenotype. Further, GAD<sub>65</sub>-specific T cells with regulatory characteristics might be able to suppress autoreactive responses and inflammation in the pancreas.



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## Abbreviations

APC	allophycocyanin
CCR	chemokine receptor, CC
CD	cluster of differentiation
cDNA	complementary DNA
C-peptide	connecting peptide
C <sub>T</sub>	comparative threshold
CTLA-4	cytotoxic T lymphocyte associated antigen
CVB4	coxsackievirus B4
CXCR	chemokine receptor, CXC
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
GABA	γ amino butyric acid
GAD <sub>65</sub>	glutamic acid decarboxylase 65
GADA	glutamic acid decarboxylase autoantibody
HLA	human leukocyte antigen
HSP	heat shock protein
IA-2A	tyrosine phosphatase autoantibody
IAA	insulin autoantibody
ICA	islet cell autoantibody
IFN	interferon
IL	interleukin
IP-10	interferon-γ induced protein-10
LADA	latent autoimmune diabetes in adults
MCP-1	monocyte chemoattractant protein-1
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
NOD mouse	non obese diabetic mouse
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll
PHA	phytohemagglutinin
RANTES	regulated upon activation normal T cell expressed and secreted
RT	reverse transcription
T1D	type 1 diabetes
T <sub>c</sub>	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
Treg	regulatory T cells

## Original publications

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

- I. **Hedman M**, Ludvigsson J, Faresjö M.K.  
Nicotinamide reduces high secretion of IFN- $\gamma$  in high-risk relatives even though it does not prevent type 1 diabetes.  
*Journal of Interferon & Cytokine Research* 2006; 26:207-213
  
- II. **Hedman M**, Faresjö M, Axelsson S, Ludvigsson J, Casas R.  
Impaired CD4<sup>+</sup> and CD8<sup>+</sup> T cell phenotype and reduced chemokine secretion in recent-onset type 1 diabetic children.  
*Clinical & Experimental Immunology* 2008; 153:360-368
  
- III. Axelsson S, **Hjorth M**, Åkerman L, Ludvigsson J, Casas R.  
Early induction of GAD<sub>65</sub>-specific T helper 2 response in type 1 diabetic children treated with alum-formulated GAD<sub>65</sub>.  
*Submitted*
  
- IV. **Hjorth M**, Axelsson S, Rydén A, Faresjö M, Ludvigsson J, Casas R.  
GAD-alum treatment induces GAD<sub>65</sub>-specific CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells and reduces CD4<sup>+</sup> cell activation in type 1 diabetic patients.  
*Submitted*

## Review of the literature

### Introduction to type 1 diabetes

Type 1 diabetes (T1D) is a chronic and serious disease usually affecting children from a young age. Autoimmune destruction of the insulin producing pancreatic  $\beta$ -cells results in insulin deficiency (Bach 1994). Insulin is released in response to increased levels of glucose in the blood after a meal. This hormone makes it possible for glucose to enter the cells, which leads to a decrease in blood glucose level. At the time of diagnosis of T1D, a majority of the  $\beta$ -cells have been destroyed by the immune system and therefore, the blood glucose level remains high due to partial insulin deficiency (Agardh *et al.* 2002). This leads to symptoms such as increased urination and thirst, fatigue and weight loss. Severe lack of insulin can lead to production of ketone bodies and acidosis with symptoms such as nausea, pain in the stomach, hyperventilation, blurred consciousness and perhaps even unconsciousness and diabetic coma, which is a dangerous acute complication. Treatment of T1D can cause hypoglycemia, which is a rather common, acute complication but can also cause late complications e.g. retinopathy, nephropathy, neuropathy and atherosclerosis (Agardh *et al.* 2002). Reasonable quality of life can be acquired with modern insulin therapy, but prevention of acute and late serious complications is facilitated by preservation of residual insulin secretion. Preventing  $\beta$ -cell destruction and thereby preserving the endogenous insulin production is an important goal of T1D therapy. Several promising clinical trials to save residual insulin secretion are underway. Hopefully, some of these will lead to clinically approved immune interventions or might even preventative regimens.

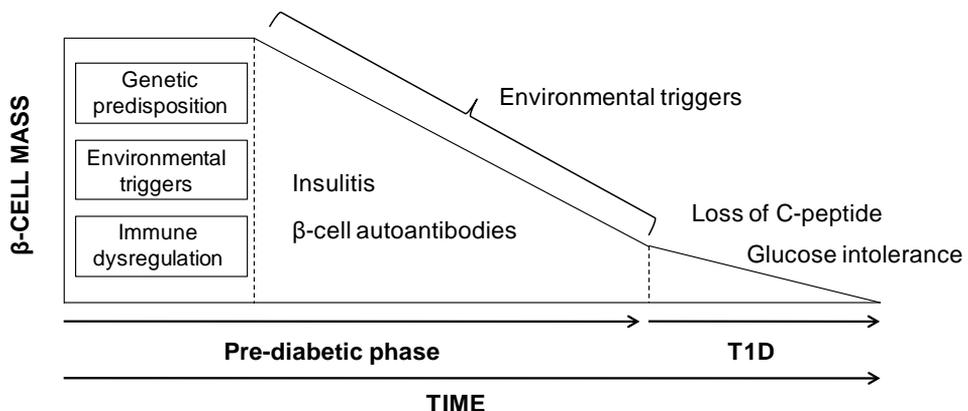
The progress of T1D in children is often rapid and the diagnosis is usually made by measurement of plasma glucose levels in combination with one or more symptoms. Children typically present with severe symptoms and the diagnosis is usually made based on non-fasting plasma glucose values  $> 12.0$  mmol/l. Rarely, fasting plasma glucose is determined and should then be  $\geq 7.0$  mmol/L for diabetes diagnosis (WHO 2006). In situations with no symptoms and undefined blood glucose values, diagnosis can also be made by oral glucose tolerance test (OGTT). Plasma glucose level of  $\geq 11.1$  mmol/L, two hours after an oral glucose load, is considered as cut-off for diabetes diagnosis (WHO 2006).

## Epidemiology

The incidence of T1D is increasing all over the world by 3-5% per year, but there is a considerable variation in different geographic areas (Green and Patterson 2001; DIAMOND 2006). Finland has the highest incidence with 64/100 000 diagnosed annually, followed by Sweden with now more than 40/100 000 new cases per year (TEDDY 2008). These high-incidence countries are in sharp contrast to China and Venezuela where less than 1/100 000 annually develop T1D (DIAMOND 2006). In parallel with an increased incidence, the median age of disease onset has decreased in Sweden during 1983-1998 (Pundziute-Lycka *et al.* 2002).

## Aetiology

The aetiology of T1D is largely unknown, but genetic and environmental factors in combination with a dysregulated immune system seem to have important roles in the autoimmune process leading to disease (Figure 1). It is believed that the autoimmune process may be initiated years before clinical onset of T1D. As a result of the immune dysregulation, circulating autoantibodies directed against pancreatic antigens can be detected in peripheral blood, which enables identification of individuals at risk of developing T1D. This gives a window of opportunity to prevent the disease in high-risk individuals.



**Figure 1. Schematic illustration of the pathogenesis and development of type 1 diabetes (T1D).** Interaction between genetic predisposition, environmental triggers and a dysregulated immune system, may induce an autoimmune response leading to loss of  $\beta$ -cells and progression to T1D (modified from Atkinson & Eisenbarth, 2001).

## Factors contributing to T1D

### **Genetics**

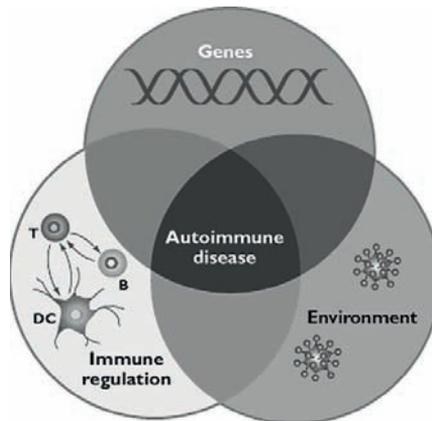
Several genes have been shown to predispose for T1D. The genetic susceptibility for T1D is mainly related to the human leukocyte antigen (HLA) class II genes DQ and DR (Atkinson and Maclaren 1994; Noble *et al.* 1996). The HLA complex provides 40-50% of the inheritable risk (Noble *et al.* 1996). Interestingly, certain HLA genotypes that conferred no risk 20 years ago are today seen in children who get diabetes (Lernmark, 2009 oral communication). Polymorphisms in the cytotoxic T lymphocyte associated antigen (CTLA)-4 gene may be more common in individuals affected by T1D than in the general population (Nistico *et al.* 1996). Mutations in the protein tyrosine phosphatase non-receptor type 22 (PTPN22) and insulin gene region have also been associated with T1D (Bain *et al.* 1992; Hermann *et al.* 2006). The PTPN22 gene encodes a lymphoid-specific phosphatase (LYP), which is an important suppressor of T cell activation (Bottini *et al.* 2004).

However, genes as a single predisposing factor are not enough for T1D to develop. Exogenous factors have a critical role in the disease process. For example, the incidence has increased over the last 50 years, faster than changes in genotype can account for. Further, only about 10% of individuals with HLA-risk genotype progress to clinical disease and the concordance of T1D in monozygotic twins is only about 40% (Kyvik *et al.* 1995; Knip *et al.* 2005). Interestingly, the disease incidence has increased in population groups who have moved from a low-incidence to a high-incidence region (Knip *et al.* 2005). These observations strongly indicate the importance of environmental factors.

### **Infections**

Viral infections, e.g. the enterovirus coxsackievirus B4 (CVB4) and cytomegalovirus, have been suggested as risk factors for development of T1D (WHO 1999). Post-mortem examination of a newly diagnosed T1D patient revealed the presence of CVB4 in the pancreas (Yoon *et al.* 1979). It has also been reported that simultaneous development of T1D in a woman and her son was associated with coxsackievirus infections in the whole family (Hindersson *et al.* 2005). Further, impaired immune responses towards CVB4 in children with T1D, could indicate that they are more prone to CVB4 infections and related complications, such as  $\beta$ -cell damage (Skarsvik *et al.* 2006). Gestational enterovirus infection has been associated with increased risk for the offspring to develop T1D (Elfving *et al.* 2008).

During the last years it has been proposed that increased hygiene with less exposure towards bacteria and viruses may cause changes in the gut bacterial flora, which influences the maturation of the immune system (Rook and Brunet 2005; Ludvigsson 2006). Reduced microbial exposure may lead to a defective regulation and imbalance of the immune system with subsequent development of allergy and autoimmunity (Rook and Brunet 2005). The incidences of allergic disorders and T1D correlate closely in the world (Stene and Nafstad 2001).



**Figure 2.** Factors that have been related to development of autoimmune disease. (Ermann and Fathman 2001).

### **Diet**

The constitution of the diet affects the gut immune system and has also been associated with development of  $\beta$ -cell autoimmunity. Short breast feeding and early introduction of cow's milk, late introduction of gluten, low vitamin D intake and high intake of nitrate have been considered as dietary triggers in children (Sadauskaite-Kuehne *et al.* 2004; Vaarala 2004; Wahlberg *et al.* 2006).

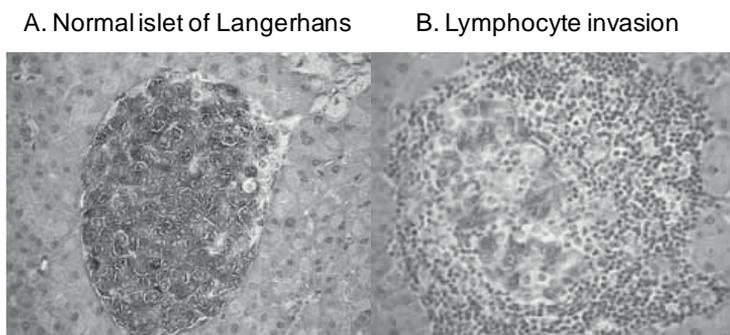
### **$\beta$ -cell stress**

$\beta$ -cell stress has been linked to development of  $\beta$ -cell autoimmunity. Increased insulin demand during periods of rapid growth, such as during infancy and puberty, could lead to an excessive workload on the  $\beta$ -cells to be able to produce enough insulin, which may trigger the autoimmune process leading to T1D (Ludvigsson 2006). The accelerator hypothesis suggests

that rapid weight gain in young children in combination with increased insulin demand could lead to insulin deficiency and T1D (Fourlanos *et al.* 2008; Ljungkrantz *et al.* 2008). Psychosocial stress may also increase the need for insulin and affect  $\beta$ -cell related autoimmunity in young children (Sepa *et al.* 2005). However, the biological mechanisms are still poorly understood.

## Immunology of type 1 diabetes

T1D is a chronic and progressive autoimmune disease caused by an inability of the body to distinguish self from non-self (Yi *et al.* 2006). A misdirected T cell-mediated attack against the islets of Langerhans in the pancreas leads to the selective destruction of insulin producing  $\beta$ -cells (Atkinson *et al.* 1994). The islets are infiltrated by T cells, B cells, macrophages, and natural killer cells, a condition commonly referred to as insulinitis (Imagawa *et al.* 1999; Moriwaki *et al.* 1999) (Figure 3). The destruction is executed by cytotoxic effector T cells using perforin and granzyme and/or interaction between Fas on  $\beta$ -cells and Fas ligand on infiltrating cells, but also by releasing cytokines, for example interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF) (Andersen *et al.* 2006). At the time of clinical symptoms, a major part of the islets are deficient in  $\beta$ -cells (Lernmark *et al.* 1995; Butler *et al.* 2007) and the total pancreatic volume is significantly reduced compared with control individuals (Lohr and Kloppel 1987). This thesis is mainly focused on the T cell population and in particular T helper CD4<sup>+</sup> cells and cytotoxic CD8<sup>+</sup> T cells, which are described in the section: T cells.



**Figure 3.** (A) Normal islet of Langerhans, stained for stored insulin in the  $\beta$ -cells. (B) Lymphocyte invasion in a prediabetic animal, with loss of stored insulin. (McDuffie Lab. University of Virginia Health System. Department of Microbiology.)

## **Autoantigens and autoantibodies**

Autoantibodies against  $\beta$ -cell antigens can be detected years before the clinical onset of T1D. However, their role in human disease is unclear. Islet cell autoantibodies (ICA) were the first T1D associated autoantibodies detected in human pancreas sections by immunofluorescence (Bottazzo *et al.* 1974). Today, the major autoantibodies used for prediction of T1D are those directed against glutamic acid decarboxylase (GADA), insulin (IAA), insulinoma associated protein-2/tyrosine phosphatase (IA-2A) and lately also zinc transporter 8 (ZnT8) (Palmer *et al.* 1983; Baekkeskov *et al.* 1990; Payton *et al.* 1995; Wenzlau *et al.* 2007). ZnT8 is a secretory granule membrane protein in pancreatic  $\beta$ -cells and has been identified as an autoantigenic target in T1D (Wenzlau *et al.* 2007). The presence of multiple antibodies can be used to identify individuals at high risk of developing T1D (Taplin and Barker 2008). About 95% of T1D patients express at least one autoantibody, while approximately 80% express two or more autoantibodies (Gottlieb and Eisenbarth 1998). Only about 2-3% of healthy individuals have autoantibodies to GAD<sub>65</sub>, insulin or IA-2 (Notkins and Lernmark 2001; Schlosser *et al.* 2004).

### ***Insulin***

Insulin was the first  $\beta$ -cell antigen detected in newly diagnosed T1D patients and insulin autoantibodies are often the first to appear in individuals developing T1D (Palmer *et al.* 1983; Ziegler *et al.* 1999). The frequency of patients positive for IAA and GADA has been shown to be higher in children from areas with high incidence of T1D (Holmberg *et al.* 2006). In newly diagnosed patients, 40-70% have IAA (Williams *et al.* 2003; Holmberg *et al.* 2006) while only about 2.5% are IAA positive in the general population (Schlosser *et al.* 2004).

### ***Glutamic acid decarboxylase***

Glutamic acid decarboxylase (GAD) is found in pancreatic islet cells and in the brain (Leslie *et al.* 1999). GAD is an enzyme involved in the conversion of glutamic acid to the inhibitory neurotransmitter  $\gamma$ -amino butyric acid (GABA) (Roberts and Frankel 1951). The physiological role of GAD in the pancreatic islets is unknown but it has been suggested that GAD may function as a negative regulator of insulin secretion in response to glucose (Shi *et al.* 2000; Notkins and Lernmark 2001). GAD was originally detected as a 64 kDa protein in plasma from T1D patients (Baekkeskov *et al.* 1982). Further studies showed that antibodies in sera from newly diagnosed T1D patients were directed against this pancreatic islet cell protein (Baekkeskov *et al.* 1987), which later was identified as the enzyme GAD<sub>65</sub> (Baekkeskov *et al.*

1990). GAD<sub>65</sub> autoantibodies (GADA) are detected in 50-80% of patients near the onset of T1D and in less than 3% of the general population (Bonifacio *et al.* 1995; Leslie *et al.* 1999; Notkins and Lernmark 2001). The levels of GADA generally increase with age (Lohmann *et al.* 1997). As a segment of GAD<sub>65</sub> (a.a 247-279) shares sequence similarity with a peptide of the coxsackievirus, this infection may be an environmental trigger of T1D (Ellis *et al.* 2005). Thus, autoimmunity in T1D may arise by molecular mimicry between GAD<sub>65</sub> and a peptide of coxsackievirus.

### ***Tyrosine phosphatase-like protein***

Additional analyses of the 64 kDa protein, identified as GAD<sub>65</sub>, revealed two other antigenic targets, the 40 and 37 kDa proteins that bound antibodies strongly associated with progression to T1D (Hawkes *et al.* 1996). The 40 kDa antigen has been identified as the tyrosine phosphatase-like protein IA-2 (ICA512), whereas the 37 kDa antigen has been suggested to be a different protein with structural similarity to IA-2 (Payton *et al.* 1995; Lu *et al.* 1996). IA-2 is localised in the secretory granule membranes of islets and other neuroendocrine cells (Solimena *et al.* 1996; Zhang *et al.* 1997). Autoantibodies against IA-2 (IA-2A) are directed to the intracellular part of the protein (Kawasaki *et al.* 1997). In newly diagnosed T1D patients, IA-2A are detected in 55-75% and in only 0-2.5% of the normal population (Bonifacio *et al.* 1998; Leslie *et al.* 1999).

### **T cells**

The precursors of T cells mature in the thymus during the process of positive and negative selection. Cells with T cell receptors (TCR) that recognise and bind antigen presented by HLA molecules will survive positive selection, while the others will die. However, if the TCR binds strongly to the autoantigen, the cell will instead die by negative selection, which is essential for maintaining tolerance to self (Janeway *et al.* 2005). The T cells are supposed to recognise only foreign peptides presented by HLA.

T cells, surviving the selection process, leave the thymus and circulate continually from the blood to peripheral lymphoid tissues (lymph nodes, spleen and mucosal tissues). When antigen presenting cells (APC) recognise antigen in the periphery, they transport it to a lymph node where the antigen is presented to T cells. The T cells with a receptor specific for the antigen will bind and then proliferate and differentiate into an effector T cell (Janeway *et al.* 2005). Cytotoxic T cells recognising peptides from intracellular pathogens, presented by HLA

class I molecules, kill the pathogen infected cells. Apoptosis is induced by interaction between Fas receptors on target cells and Fas ligand on infiltrating cells, but also by the cytotoxic effects of perforin and granzyme (Janeway *et al.* 2005).

## **T cell subpopulations and cytokines**

T cells can be defined by expression of the cell surface and co-stimulatory molecule cluster of differentiation (CD) 3. The T cells can be further classified into different subpopulations based on their expression of CD4 and CD8, which defines T helper (Th) and cytotoxic T cells (Tc), respectively (Janeway *et al.* 2005). The Th cells can be divided into Th1 and Th2 cells based on their cytokine profile (Mosmann *et al.* 1986; Del Prete *et al.* 1991). Cytokines act as intercellular mediators in co-ordinating cellular responses. In mice, it has been shown that Th1 cells produce IFN- $\gamma$ , interleukin (IL)-2 and TNF, which are responsible for activating macrophages and Tc cells at the site of infection and are thereby important in cell-mediated immunity against intracellular pathogens (Mosmann and Sad 1996). The Th2 cells secrete IL-4, -5 and -13 that are important in the humoral immune response, since they activate eosinophils and induce antibody production by B cells to eliminate extracellular pathogens (Mosmann and Sad 1996). However, in humans the cytokine production is not as tightly restricted to a single subset as in the mouse. Besides their different tasks in the immune system, Th1 and Th2 cells stimulate the development of their own subset while they inhibit the opposing one (Mosmann and Sad 1996; O'Garra *et al.* 1997). Another important subset of T cells is the regulatory T cells (Treg) that will be described in the section: regulatory T cells.

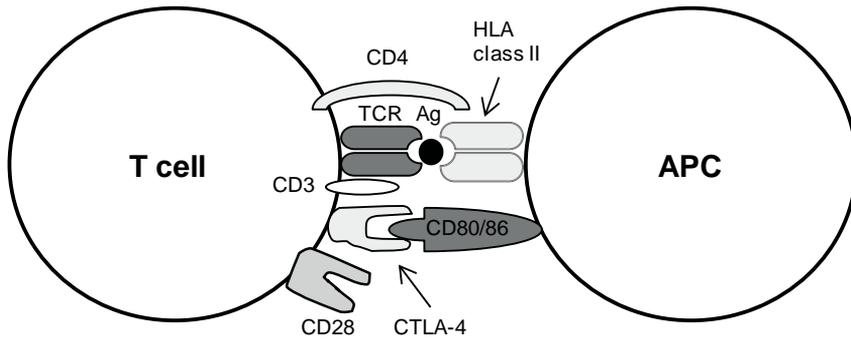
An imbalance between Th1 and Th2 associated cytokines have been suggested to be of importance in mediating the  $\beta$ -cell destruction, seen in T1D (Tisch and McDevitt 1996). The cells infiltrating human islets produce Th1 cytokines like IFN- $\gamma$  (Foulis *et al.* 1991). Regulatory Th3 cells producing transforming growth factor (TGF)- $\beta$  inhibit the differentiation of naïve CD4<sup>+</sup> cells into the Th1 cell lineage, which results in suppression of the autoimmune process (Schmitt *et al.* 1994; Weiner 2001). Cytokines can also be classified as pro-inflammatory (for example IL-1, IL-6 and TNF) and anti-inflammatory (for example IL-4 and IL-10) (Dinarello 2000).

A subset of IL-17 producing T cells (Th17) cells has received a lot of attention during the last few years. This subpopulation has a crucial role in the induction of tissue injury by producing pro-inflammatory cytokines, such as IL-17A, IL-17F and IL-22 (Kolls and Linden 2004). This

leads to a substantial tissue response due to the wide distribution of IL-17 and IL-22 receptors (Kurts 2008; Korn *et al.* 2009; Lee *et al.* 2009). Interleukin-17A promotes tissue inflammation by induction of other pro-inflammatory cytokines and chemokines, which in turn attract and activate granulocytes and macrophages (Kolls and Linden 2004; Weaver *et al.* 2007). It has been suggested that TGF- $\beta$  together with IL-1 $\beta$ , IL-6 and IL-21 are necessary for up-regulation of the transcription factor RORC, which is required for the induction of IL-17 production in human naïve T cells (Yang *et al.* 2008). Increased levels of IL-17 have been observed in various autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, inflammatory colitis, rheumatoid arthritis and T1D compared with healthy controls (Bettelli *et al.* 2007; Bradshaw *et al.* 2009).

### **The immunological synapse**

Naïve T cells become activated after antigen recognition. The antigen is presented on HLA class II molecules by APC and the TCR on T cells recognises the HLA-peptide complex and delivers the first signal for T cell activation via CD3. (Figure 4). T cells need additional co-stimulatory signals to become activated (Iezzi *et al.* 1998). CD28 is expressed on T cells and binds CD80/86 (B7 molecules) on APCs. CD28 ligation of naïve T cells is required for IL-2 production and cell proliferation (Acuto and Michel 2003). Most activated memory T cells maintain their surface expression of CD28, suggesting that it is also important in reactivation of T cells (Sharpe and Freeman 2002). Other co-stimulatory molecules have been described, such as ICOS, CD134 (OX40) and CD27. The CTLA-4 protein is primarily expressed in endosomal compartments and is up-regulated on the T cell surface after activation (Perkins *et al.* 1996; Wang *et al.* 2001). CTLA-4 can out-compete CD28 for ligation of CD80/86 due to higher affinity, leading to down-regulation of T cell responses, which is necessary to avoid damage in the surrounding tissue. Hence, CD28 has an important role in promoting T cell responses, while CTLA-4 acts as an inhibitor (Alegre *et al.* 2001; Sharpe and Freeman 2002). The importance of CTLA-4 has been shown in knockout mice, which die within four weeks of birth from lymphoproliferative disease (Tivol *et al.* 1995).

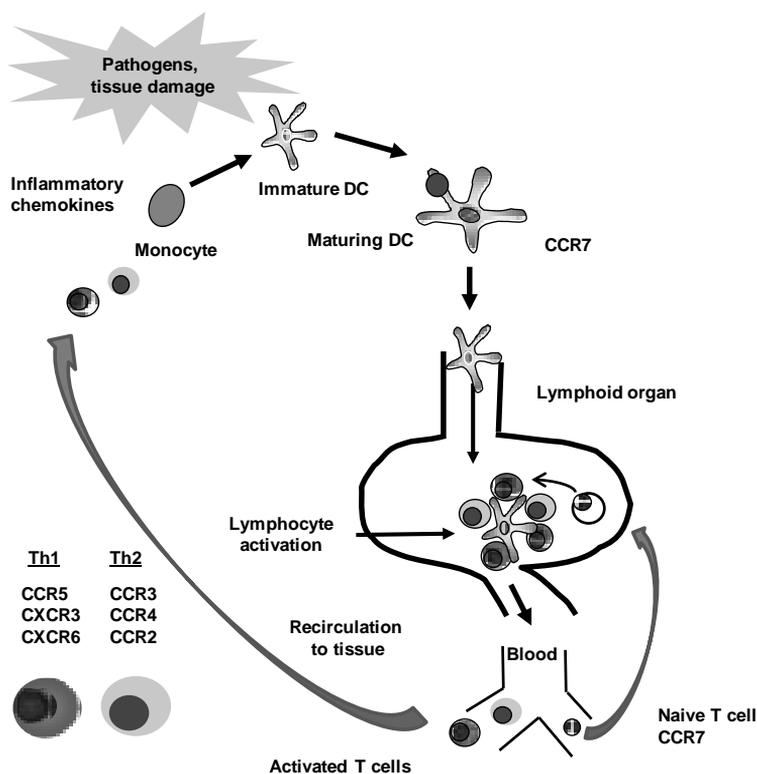


**Figure 4. Schematic illustration of the immunological synapse.**

The antigen presenting cells (APC) presents a peptide bound to human leukocyte antigen (HLA) class II. The T cell receptor (TCR) on T cells recognises the HLA-peptide complex and delivers the first signal for T cell activation via CD3. The T cell receives a second signal through the binding of CD28-CD80/86. Upon activation, cytotoxic T lymphocyte associated antigen (CTLA-4) is up-regulated and out-competes CD28 in binding affinity to CD80/86. Hence, an inhibitory signal is conferred.

## Chemokines and chemokine receptors

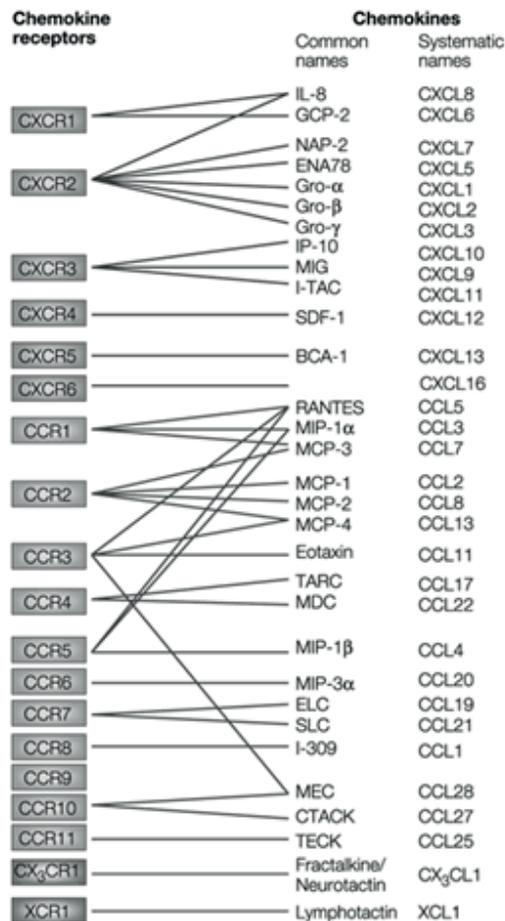
Lymphocytes continuously traffic from blood to lymph nodes and back again to the blood via the efferent lymphatics and the thoracic duct (Cyster 2003). Despite this continuous traffic, the size of lymph nodes usually remains constant, indicating that the rates of cell entry and exit are normally in equilibrium (Sallusto and Mackay 2004). This process is regulated by chemokines and their receptors, selectins and integrins, which are important mediators of this communication as they enable leukocyte migration during homeostasis but also during inflammation (Sallusto and Mackay 2004). The chemokines are small peptides with chemoattractant properties that are produced by a variety of cells in response to bacteria, viruses or tissue damage (Atkinson and Wilson 2002) (Figure 5). The chemokines induce cytokine secretion and direct leukocyte migration along a gradient of chemokine molecules, which increases in concentration towards the site of infection.



**Figure 5. Schematic overview of migration of leukocytes in response to pathogens or tissue damage. (Modified from Sallusto and Lanzavecchia 2000)**

The differentiation of  $CD4^+$  cells is accompanied by the acquisition of chemokine receptors and migratory abilities under the influence of chemokines. The Th1 and Th2 cell subsets have been defined based on their chemokine receptor expression. Th1 cells preferentially express CCR5, CXCR3 and CXCR6, while Th2 cells express CCR4 and CCR8 (Bromley *et al.* 2008). This is the *in vitro* scenario, which mainly has been studied in animal models. *In vivo*, the Th cells show a more complex chemokine receptor profile (Bromley *et al.* 2008). Freshly isolated T cells express both the Th1-associated receptors CXCR3 and CCR5 and the Th2 receptor CCR4 (Andrew *et al.* 2001), which indicate that accurate identification of T cell subsets requires additional markers. In this thesis, our purpose for analysing chemokines and their receptors was to characterise different cell populations in recent-onset T1D patients and not to investigate migratory pathways and signalling mechanisms. We were especially interested in  $CD8^+$  cells, which are central in the destructive process of insulinitis.

Chemokines interact with their receptors, which are seven transmembrane domain G protein-coupled rhodopsin-like receptors expressed on the cell surface (Janeway *et al.* 2005). There are two main groups of chemokine receptors: CC and CXC. The largest group of chemokine receptors is the CC group with two adjacent cysteines in the N-terminus. The CXC group has a variable amino acid between the two N-terminal cysteins. More chemokines than chemokine receptors are known today, suggesting promiscuity of the receptors. In this thesis, the expression of the chemokine receptors CXCR3, CXCR6, CCR4, CCR5 and CCR7 have been studied and are therefore discussed here. A list of chemokines and their receptors is shown in figure 6.



**Figure 6. Chemokine receptors and their chemokine ligands (Proudfoot 2002).**

CXCR3 is expressed on naïve and activated CD4<sup>+</sup> and CD8<sup>+</sup> cells and natural killer (NK) cells (Loetscher *et al.* 1996; Guarda *et al.* 2007). The receptor is activated by the three IFN- $\gamma$  inducible chemokines CXCL9/MIG (monokine induced by IFN- $\gamma$ ), CXCL10/IP-10 (IFN- $\gamma$  inducible protein), and CXCL11/ITAC (IFN inducible T cell  $\alpha$  chemoattractant), which suggests a role of CXCR3 in Th1 immune responses (Cole *et al.* 1998; Loetscher *et al.* 1998; Weng *et al.* 1998). These chemokines are up-regulated in a pro-inflammatory cytokine milieu and their major function is to selectively recruit immune cells to inflammation sites. CXCR3 is involved in the development of autoimmune diseases, especially by creating local amplification of inflammation in target organs (Lacotte *et al.* 2009).

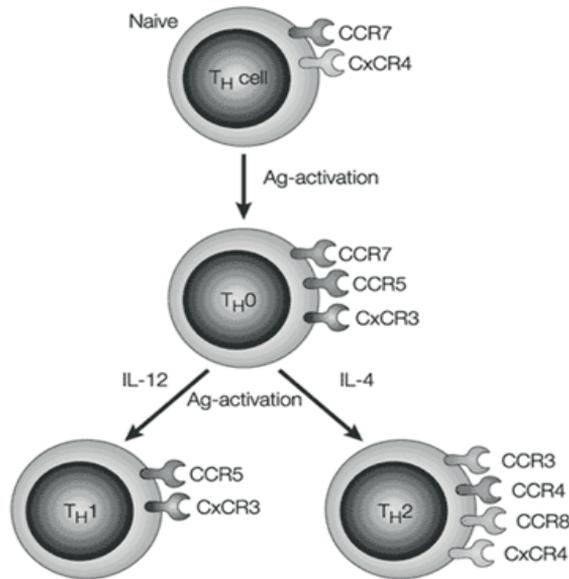
CXCR6 is expressed on memory T cells and activated Th1 and Tc1 effector cells (Unutmaz *et al.* 2000; Kim *et al.* 2001). After recruitment into inflamed tissues and antigen activation, T cells lose the expression of CXCR6 and accumulate at the inflammation site where they produce large amounts of IFN- $\gamma$  (Koprak *et al.* 2003). CXCR6 binds the chemokine CXCL16 (Matloubian *et al.* 2000). The biological role of CXCR6 has been suggested to involve migration of Th1 and Tc1 cells into sites of inflammation (Kim *et al.* 2001).

CCR4 is expressed on Th2 cells (Sallusto *et al.* 1998; Syrbe *et al.* 1999) and is important for dendritic cell migration and T cell recirculation from tissue to draining lymph nodes (Sozzani *et al.* 1999). The chemokines CCL17/TARC (thymus and activation regulated chemokine) and CCL22/MDC (monocyte-derived chemokine) bind to CCR4 (Imai *et al.* 1999).

CCR5 is expressed on several different cell types, for example activated Th and Tc cells and naïve CD8<sup>+</sup> cells (Bromley *et al.* 2008). The receptor binds a number of different chemokines including CCL3/MIP (macrophage inflammatory protein)-1 $\alpha$ , CCL4/MIP-1 $\beta$  and CCL5/RANTES (regulated on activation normal T cell expressed and secreted) (Samson *et al.* 1996). The expression of CCR5 has been associated with migration of Th1 cells and lately also Treg cells (Bonecchi *et al.* 1998; Zhang *et al.* 2009).

CCR7 is involved in migration and recirculation of naïve T and B cells, Treg cells, central memory T (T<sub>CM</sub>) cells and dendritic cells across high endothelial venules into secondary lymphoid organs (Sallusto *et al.* 1999; Reif *et al.* 2002; Szanya *et al.* 2002; Ohl *et al.* 2004; Forster *et al.* 2008). CCR7<sup>+</sup> T<sub>CM</sub> cells lack direct effector function, but efficiently stimulate dendritic cells and differentiate into CCR7<sup>-</sup> effector cells upon secondary stimulation. CCR7

binds to the ligands CCL19/MIP-3 $\beta$  and CCL21/6CKine (Rot and von Andrian 2004; Forster *et al.* 2008). The expression of most chemokines is induced during infection and inflammation, but some chemokines, including CCL19 and CCL21, are constitutively expressed and control cell movement during homeostasis (Rot and von Andrian 2004).



**Figure 7. Expression pattern of chemokine receptors on T helper (Th) cells is related to the differentiation state of the cells. Naïve cells express CCR7 and CXCR4 that participate in entry of the lymph node through high endothelial venules. Recently activated cells maintain CCR7 expression, but also express CCR5 and CXCR3. Antigen stimulation in the presence of Th1 or Th2 like cytokines alters the chemokine receptor pattern. Th1 associated cells express CCR5 and CXCR3 while Th2 cells induce CCR3, CCR4, CCR8 and CXCR4 (Lukacs 2001).**

The promiscuity between chemokines and their receptors suggests a complex regulatory network with agonistic and antagonistic effects depending on the unique chemokine/chemokine receptor combination (Blanpain *et al.* 1999; Loetscher *et al.* 2001; Ogilvie *et al.* 2001; Colobran *et al.* 2007).

### Receptors expressed on T cells

Cytokine receptors are essential for cell communication. Many of the cytokine receptors are members of the class I hematopoietin superfamily, which the interleukin receptors belong to

(Janeway *et al.* 2005). The IL-12 receptor (IL-12R) is expressed on activated T cells and NK cells (Presky *et al.* 1996). Its expression is dependent on the transcription factor T-bet, which is up-regulated by IFN- $\gamma$  (Afkarian *et al.* 2002). The IL-12R is inhibited by IL-4 and therefore, its expression is associated with Th1 cells (Rogge *et al.* 1997). The IL-18 receptor (IL-18R) binds the pro-inflammatory cytokine IL-18, which is important in maturation of effector CD8<sup>+</sup> cells (Kohyama *et al.* 1998). Combined IL-12 and IL-18 secretion greatly induces the production of IFN- $\gamma$  by T, B, NK and dendritic cells and inhibits IL-4 dependent antibody production of B cells (Yoshimoto *et al.* 1998).

The highly glycosylated enzyme CD45 antigen is also called protein tyrosine phosphatase receptor type C (PTPRC) (Kaplan *et al.* 1990). The CD45 family consists of multiple members, among them CD45RA and CD45RO, which have been studied in this thesis. CD45RA is located on naïve T cells, while CD45RO is expressed on memory T cells.

## **Regulatory T cells**

Regulatory T cells (Treg) constitute a subpopulation of T cells that have received considerable attention as key players of tolerance to self-antigens (Sakaguchi *et al.* 1995; Bellinghausen *et al.* 2003). The thymically derived naturally occurring Tregs (nTreg) constitute 1-6% of the total peripheral CD4<sup>+</sup> T cell population, have low proliferative capacity and down-regulate immune responses (Sakaguchi *et al.* 1995; Shevach *et al.* 2001; Bellinghausen *et al.* 2003). Suppressor function requires TCR activation, but once activated their function can be non-specific (Brusko *et al.* 2008). The suppression by nTreg is mediated by a cell contact dependent mechanism, for example by inhibiting IL-2 production in responder cells or possibly by secretion of IL-35 (von Boehmer 2005; Collison *et al.* 2007).

The majority of human Tregs are found within the CD4<sup>+</sup> cell subset expressing high levels of the IL-2 receptor  $\alpha$ -chain; CD25, hence termed CD4<sup>+</sup>CD25<sup>high</sup> (Baecher-Allan *et al.* 2001; Liu *et al.* 2006). However, the CD4<sup>+</sup>CD25<sup>high</sup> cell population is functionally and phenotypically heterogeneous, since it contains both suppressor and effector cells (Baecher-Allan *et al.* 2005). Molecular markers that accurately can define suppressive Tregs are therefore needed to distinguish them from activated cells.

At present, the transcription factor Forkhead box p3 (FOXP3) is the best marker for Tregs (Hori *et al.* 2003; Ziegler 2006). FOXP3 is involved in Treg lineage commitment and controls

a number of essential functions of Tregs, for example transcriptional suppression of cytokines and up-regulation of CD25, CTLA-4 and TGF- $\beta$  expression (Yagi *et al.* 2004; Bettelli *et al.* 2005; Serfling *et al.* 2006). The expression of FOXP3 is associated with the suppressive function of Tregs, but its expression can be up-regulated by *in vitro* activation of non-Tregs (Walker *et al.* 2003; Allan *et al.* 2007; Wang *et al.* 2007), which makes identification of Tregs difficult. However, the expression is transient and disappears after a number of days (Gavin *et al.* 2006b; Pillai *et al.* 2007). The constitutive expression of FOXP3 in Tregs has been suggested to be regulated by epigenetics and new techniques to detect “true” Tregs are underway (Baron *et al.* 2007; Floess *et al.* 2007). Low expression of the IL-7 receptor  $\alpha$  chain, CD127, in combination with FOXP3 and CD25 expression correlate with suppressive function (Liu *et al.* 2006; Seddiki *et al.* 2006). Activated T cells rapidly express CD127 and memory T cells express high levels of the protein, but the Treg cell population remains CD127<sup>low</sup> (Liu *et al.* 2006). The combined expression of GITR (glucocorticoid-induced tumour necrosis factor receptor), CD62L, and programmed cell death (PD)-1 has also been used for the discrimination of Tregs from activated non-regulatory T cells (Brusko *et al.* 2008). Lately, CD45RA has been shown to discriminate resting Treg (CD45RA<sup>+</sup>FOXP3<sup>low</sup>) from activated Treg (CD45RA<sup>-</sup>FOXP3<sup>high</sup>) (Miyara *et al.* 2009).

Neuropilin-1 has been reported to be constitutively expressed on the surface of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in mice, independent of activation status, while its expression was down-regulated in naïve CD4<sup>+</sup>CD25<sup>-</sup> cells after TCR stimulation (Bruder *et al.* 2004). Based on the finding that CD4<sup>+</sup>Neuropilin-1<sup>high</sup> cells also expressed high levels of FOXP3 mRNA and suppressed CD4<sup>+</sup>CD25<sup>-</sup> T cells, Neuropilin-1 was proposed as a useful surface marker to distinguish Treg cells from naïve and recently activated CD4<sup>+</sup>CD25<sup>+</sup> non-regulatory T cells. This made us interested to study its expression in samples from the GAD<sub>65</sub>-vaccination study (paper IV). However, we and others have observed that Neuropilin-1 cannot be used as a specific marker of human Treg (paper IV; Milpied *et al.* 2009). Instead, its expression is more likely to be related to T cell activation.

Adaptive CD4<sup>+</sup>CD25<sup>+</sup> Tregs are induced in the periphery from CD4<sup>+</sup>CD25<sup>-</sup> T cells by antigenic stimulation in the presence of a certain cytokine environment, such as IL-10 and TGF- $\beta$ , to become CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells (Chen *et al.* 2003; Karim *et al.* 2004; Taams and Akbar 2005). Other types of Tregs have been described, including Tr1 and Th3 cells exerting their effect by IL-10 and TGF- $\beta$  (Weiner 2001; Levings and Roncarolo 2005).

Interleukin-10 inhibits cytokine secretion and down-regulates MHC class II and CD80/86 expression on APC, thereby suppressing T cell responses to antigen (Moore *et al.* 2001). Since TGF- $\beta$  is broadly expressed and acts on multiple cell types, Th3 cells probably have a major role in many aspects of immune regulation and T cell homeostasis (Gorelik and Flavell 2002). Proliferation of T cells, their production of cytokines and cytotoxic effects are inhibited by TGF- $\beta$ .

### ***Tregs in human autoimmune disease***

Despite the process of positive and negative selection in the thymus, the majority of people have cells with TCRs directed towards self-antigens. Still, most people never develop autoimmune disease, partly because of the important role of Tregs in maintaining peripheral tolerance (Bach 2003). It is unclear why the presence of autoreactive T cells can be successfully regulated in healthy individuals but develop into autoimmune disease in others. An imbalanced immune system may play a major role in the pathogenesis of autoimmune disease, resulting in a deficiency in either Treg frequency or function (Chatenoud *et al.* 2001; Lindley *et al.* 2005). Another hypothesis proposes that failed immunoregulation develops when the T effector (Teff) cells overpower the capacity of Tregs to actively maintain tolerance (Torgerson 2006). Even though the general agreement today is that the frequency of Tregs is normal in individuals with autoimmune disease, some studies have reported decreased peripheral number but increased number and potency of cells isolated from inflammatory sites, which suggests an active recruitment of Treg cells to the damaged tissue (Cao *et al.* 2004; van Amelsfort *et al.* 2004).

## Immune Prevention & Intervention

Preventing  $\beta$ -cell destruction to maintain endogenous insulin production is an important goal of T1D therapy. To achieve this, the immune system needs to learn how to tolerate autoantigens but maintain a strong response against foreign antigens.

### General immunosuppressors

Previous attempts to modulate the immune system with general immunosuppressors, such as cyclosporine, azathioprine and prednisone have led to severe side-effects with increased risk of infections and cancer (Eisenbarth 1986; Bougneres *et al.* 1988; Silverstein *et al.* 1988; Atkinson and Maclaren 1994; Parving *et al.* 1999). No lasting effects on the disease were shown once the drugs were withdrawn and therefore continuous treatment to prevent progression of  $\beta$ -cell loss was required (Bougneres *et al.* 1988; Buckingham and Sandborg 2000; Herold *et al.* 2005).

### Vitamins

The inflammatory process resulting in pancreatic  $\beta$ -cell destruction is associated with elevated levels of reactive oxygen and nitric radicals, which damage cell membranes and protein structures. The protective effect of vitamins has been investigated mainly in animal models and is believed to be mediated by anti-oxidative effects (Klareskog *et al.* 2005). Vitamins have been used in several clinical trials (Pozzilli *et al.* 1995; Elliott *et al.* 1996; Pozzilli *et al.* 1997; Gale *et al.* 2004). However, no major improvement in the course of disease has been reached in trials with Nicotinamide (vitamin B<sub>3</sub>) and vitamin E (Pozzilli *et al.* 1995; Pozzilli *et al.* 1997). Combination therapy with a high oral dose of anti-oxidative agents (Nicotinamide, vitamin C, vitamin E,  $\beta$ -carotene and selenium) was tested in a double-blind placebo-controlled clinical study in newly diagnosed T1D children (Ludvigsson *et al.* 2001). Once again, no effects on the metabolic balance or preservation of  $\beta$ -cell function were observed.

However, vitamin D might be an important therapeutic target for prevention of autoimmune diabetes (Li *et al.* 2009), since studies have shown that supplementation during infancy is associated with a decreased risk of T1D (Hypponen *et al.* 2001; Stene and Joner 2003). Studies in non obese diabetic (NOD) mice, an animal model spontaneously developing autoimmune diabetes, show that the incidence of autoimmune diabetes increases when the

animals are nutritionally deprived of vitamin D (Giulietti *et al.* 2004). Instead, the disease can be prevented by vitamin D supplementation (Mathieu *et al.* 1994). The prevention seen in NOD mice may be due to combined effects of vitamin D on antigen presenting cells, activated T cells and Tregs (Mathieu *et al.* 1994; Piemonti *et al.* 2000).

### **Nicotinamide**

Nicotinamide is part of the Vitamin B group (Vitamin B<sub>3</sub>) and is classed as a food additive. Nicotinamide is a component of nicotinamide adenine dinucleotide (NAD), a coenzyme involved in many cellular oxidation-reduction reactions (Kolb and Burkart 1999). Damage of DNA in cells activates DNA repair enzymes consuming NAD, which leads to a rapid decrease in available intracellular energy levels. Thus, excessive activation of DNA repair enzymes and depletion of intracellular NAD predispose for cell death. The protective effect of Nicotinamide on  $\beta$ -cells is thought to be mediated by inhibition of this pathway by restoring NAD depots (Kolb and Burkart 1999).

Studies in NOD mice have shown protection against development of diabetes after Nicotinamide treatment (Yamada *et al.* 1982). Administration of high doses of Nicotinamide to individuals with severe acne was shown to induce anti-inflammatory actions (Niren 2006). Nicotinamide has also been tested in humans as a possible therapeutic to preserve  $\beta$ -cells and to prevent or delay the onset of T1D (Elliott and Chase 1991; Elliott *et al.* 1996; Gale *et al.* 2004). In the prospective, placebo-controlled, double-blind European Nicotinamide Diabetes Intervention Trial (ENDIT), high-risk participants were recruited from 18 European countries, Canada and USA and were randomised to daily oral Nicotinamide (1.2 g/m<sup>2</sup>) or placebo for five years (Gale *et al.* 2004). The anticipated 5-year risk of T1D was 40% (Gale 2003). More than 30 000 relatives of T1D patients were screened for islet cell antibodies (ICA). Out of these, 552 individuals with  $\geq 20$  International Juvenile Diabetes Federation (IJDF) units were included (Gale *et al.* 2004). During the trial, 159 individuals developed T1D. In Sweden, 2000 individuals were screened, 23 were included and when the study ended, seven had been diagnosed with T1D. The study revealed no differences between the groups and Nicotinamide was considered to be ineffective at the dosage used. However, previous studies in humans have shown protective effects of Nicotinamide as measured by prevention of disease in ICA positive children (Elliott and Chase 1991; Elliott *et al.* 1996) and in preservation of C-peptide in newly diagnosed T1D patients (Pozzilli *et al.* 1996).

## **Immunomodulators**

### ***Anti-CD3 monoclonal antibody***

The protein complex CD3 is found on the T cell surface and transduces signals from the TCR to start activation of the T cell. The monoclonal antibody OKT3, directed against CD3, has been shown to inhibit Tc cell mediated lysis of target cells (Chang *et al.* 1981). OKT3 has a strong mitogenic activity and induces massive amounts of cytokines (Abramowicz *et al.* 1989), leading to chills, fever, hypotension, and breathing difficulties (Thistlethwaite *et al.* 1984). These effects are caused by cross-linking of CD3<sup>+</sup> T cells and Fc receptor (FcR) bearing cells that bind to the Fc portion of the antibodies, leading to activation of both the T cell and the FcR bearing cells (Kaufman and Herold 2009).

Modified non-FcR binding CD3 antibodies have been tested in clinical trials. Newly diagnosed T1D patients were randomly assigned humanised OKT3 $\gamma$  (Teplizumab) or placebo for 14 days (Herold *et al.* 2002; Herold *et al.* 2005). A majority of the individuals that received treatment shortly after diagnosis had a maintained or improved C-peptide response still after two years, compared with the control subjects (Herold *et al.* 2005). Insulin usage was reduced and HbA1c levels were also improved. The adverse events were frequent but generally mild, even though some patients had serious adverse events. The mechanisms of modified anti-CD3 mAb in humans are not clear (Kaufman and Herold 2009). It has been shown that the number of lymphocytes declined transiently, but the number of circulating CD8<sup>+</sup> cells increased after drug treatment and persisted for an extended period of time (Herold *et al.* 2005; Keymeulen *et al.* 2005). The increase in circulating CD8<sup>+</sup> T cells was associated with reduced insulin requirements. The CD8<sup>+</sup> cells expressed FOXP3 and CTLA-4 and were found to have regulatory function (Bisikirska *et al.* 2005). It was recently reported that high-dose Teplizumab preserved the endogenous insulin secretion still five years after intervention in newly diagnosed T1D patients (Herold *et al.* 2009). However, due to more adverse events, probably as a result of the higher dosage, the enrolment of patients in that study was closed after only six patients had been treated. Further studies are ongoing with different doses.

### ***Anti-CD20 monoclonal antibody (Rituximab)***

Any evidence for B cells promoting T1D in humans has not yet been presented. However, detection of autoantibodies to islet antigens before the onset of clinical disease suggests that B

cells have a role in the immunological events leading to T1D. Data from NOD mice studies indicate that B cells are required for disease induction and are likely to have a number of roles in the pathogenesis (Serreze *et al.* 1996; Noorchashm *et al.* 1997; Wong and Wen 2005). B cells express CD20 and the anti-CD20 monoclonal antibody Rituximab selectively depletes these cells (Maloney *et al.* 1997). Treatment with CD20 monoclonal antibodies may prevent and reverse autoimmune diabetes in mice and induce regulatory cells (Hu *et al.* 2007). In humans, a clinical trial with Rituximab in new onset T1D patients has yielded promising preliminary findings (O'Neill *et al.* 2009).

### ***Interleukin-1 antagonist (Anakinra)***

Pro-inflammatory cytokines, and in particular IL-1, have been suggested to cause  $\beta$ -cell apoptosis and aggravate diabetes (Mandrup-Poulsen 1996; Eizirik and Mandrup-Poulsen 2001). Inhibition of IL-1 reduces diabetes incidence in animal models of T1D (Mandrup-Poulsen 1996). Anakinra blocks the activity of IL-1 by inhibiting its binding to the IL-1 receptor, which is expressed in a wide variety of tissues and organs (Hannum *et al.* 1990). Anakinra is approved for treatment of rheumatoid arthritis (Schiff 2004; Schiff *et al.* 2004; Fleischmann *et al.* 2006) and a clinical trial with an IL-1 $\beta$  antibody in recent-onset T1D is at present underway (Pickersgill and Mandrup-Poulsen 2009).

### **Antigen-based immunotherapy**

The hypothesis behind antigen-based immunotherapy is to restore tolerance by administration of the target antigen. Antigen-specific tolerance can be achieved by induction of anergy or induction of Treg cells (Tang *et al.* 2004; Chen *et al.* 2005). The process of anergy is mediated by antigen presentation without appropriate co-stimulatory signals, preventing the priming of autoreactive T cells. Antigen-specific Treg cells are suggested to effectively suppress the autoreactive cells in a way that induces protective immune responses (Long *et al.* 2009). Previous results in mouse models suggest that vaccination with GAD<sub>65</sub> may induce Treg cells that specifically down-regulate existing GAD<sub>65</sub> autoreactive T cells and delay the onset of T1D (Tisch *et al.* 1998).

### ***Heat-shock protein (Hsp)***

Heat-shock proteins (Hsp) are highly conserved and immunogenic proteins found in microbes, but also in mammals (Kaufmann *et al.* 1991). These widely distributed proteins are induced by stress and protect cells from stress induced damage. The Hsps are also present in cells under normal conditions, acting as chaperones, making sure that the cell's proteins are in

the right shape and place, at the right time. Due to their conserved nature, Hsp proteins easily become the target of immune responses (Rajaiah and Moudgil 2009). Microbial Hsp can activate T cells and induce antibody production and may cross-react with the corresponding mammalian Hsp (molecular mimicry), which triggers an autoimmune response. A role of Hsp in the induction of autoimmunity has been proposed in several diseases, including atherosclerosis, rheumatoid arthritis and T1D (Birk *et al.* 1996; Abulafia-Lapid *et al.* 1999). Both diabetic mice and human patients have been shown to respond to the 437–460 peptide sequence of Hsp60, p277. Treatment of NOD mice with p277 leads to protection against diabetes (Bockova *et al.* 1997; Ablamunits *et al.* 1998). Clinical trials in T1D patients report a deviation of the immune response from Th1 to Th2 and induction of the regulatory associated cytokine IL-10, after DiaPep277 therapy (Raz *et al.* 2001; Huurman *et al.* 2008). However, the effects of  $\beta$ -cell preservation are modest and are so far only seen in adults (Eldor *et al.* 2009). Recently, a phase III trial in recent-onset T1D patients has ended its inclusion (<http://clinicaltrials.gov/ct2/show/NCT00615264?term=diapep277>).

### **Insulin**

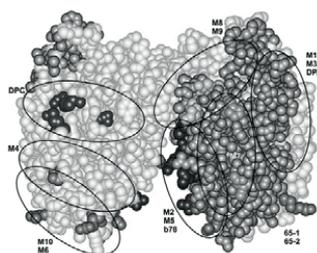
The interest in specific  $\beta$ -cell therapy is increasing. At present the two autoantigens, insulin and GAD<sub>65</sub>, have reached clinical Phase II studies and further. The effect of therapy with injected and oral insulin in relatives at risk of T1D has been studied in the Diabetes Prevention Trial of Type 1 Diabetes (DPT-1). Neither injected, nor oral insulin prevented or delayed the development of T1D (DPT-1 2002; Skyler *et al.* 2005). However, in a subgroup of individuals receiving oral insulin, with insulin autoantibodies (IAA)  $\geq 80$  nU/mL, the proportion who developed diabetes was lower compared with the placebo group (Skyler *et al.* 2005). In individuals with baseline IAA  $>300$  nU/mL, the benefit was even more dramatic, with an anticipated delay in T1D of nearly 10 years. The Type 1 Diabetes Prediction and Prevention Study (DIPP) was conducted in Finnish newborns with high-risk genotypes for T1D, and among their siblings if identified with increased risk. The participants were randomised to receive intranasal insulin or placebo. However, nasal insulin did not prevent or delay development of T1D (Nanto-Salonen *et al.* 2008).

## **Glutamic acid decarboxylase (GAD<sub>65</sub>)**

### *Effect of GAD<sub>65</sub> in non obese diabetic mice*

In 1993, studies in NOD mice demonstrated that the destruction of pancreatic islet  $\beta$ -cells was associated with T cells recognising GAD<sub>65</sub>. Further, injection with GAD<sub>65</sub> markedly reduced T cell proliferative responses to the autoantigen (Kaufman *et al.* 1993; Tisch *et al.* 1993). A few years later, it was shown that a single intranasal dose of GAD<sub>65</sub> peptides in NOD mice induced high levels of GAD<sub>65</sub> antibodies. Further, the treatment greatly reduced IFN- $\gamma$  while IL-5 responses were increased towards GAD<sub>65</sub>, thus indicating a shift from Th1 toward Th2 responses (Tian *et al.* 1996a). It was suggested that spreading of the Th2 response to other autoantigens than GAD<sub>65</sub> could create a cascade of anti-inflammatory responses (Tian *et al.* 1997). Intravenous administration of GAD<sub>65</sub> delayed the onset of diabetes and the progression of insulinitis in NOD mice (Tisch *et al.* 1998). Thus, a role of GAD<sub>65</sub>-specific Treg cells with Th2 phenotype was suggested as mediators of the suppression of the autoimmune response.

The potential therapeutic use of GAD<sub>65</sub> in human T1D began to evolve. In 1994, the biopharmaceutical company Diamyd Medical AB (Stockholm, Sweden) licensed the rights to GAD<sub>65</sub>. A wide range of animal safety studies were performed to support the clinical use of alum-formulated recombinant human (rh) GAD<sub>65</sub> (GAD-alum) prior to clinical studies in humans (Plesner *et al.* 1998; Bieg *et al.* 1999; Uibo and Lernmark 2008; Diamyd Medical, unpublished). No adverse events other than local inflammation at the injection site were observed in mice, rats, rabbits, marmosets or dogs, and all studies were reported to regulatory agencies (Uibo and Lernmark 2008). Immunisation with GAD<sub>65</sub> induced both T and B cell responses without causing insulinitis, diabetes or any neurological abnormalities (Plesner *et al.* 1998; Bieg *et al.* 1999).



**Figure 8. The GAD<sub>65</sub> molecule.** (Diamyd Medical AB, 2009)

### *GAD<sub>65</sub> formulated in alum*

GAD<sub>65</sub> is manufactured via a process involving expression in an insect cell line infected with recombinant baculovirus containing the human GAD<sub>65</sub> cDNA (Diamyd Medical AB, Stockholm, Sweden). Aluminium hydroxide (Alhydrogel®) was selected as adjuvant for formulation with rhGAD<sub>65</sub> due to the properties of aluminium salts, inducing humoral rather than cellular immune responses. It was expected that this would minimise the possibility of promoting cell mediated  $\beta$ -cell destruction (Uibo and Lernmark 2008). Further, alum is currently used in several commercial vaccines and was, until recently, the only adjuvant approved by the American food and drug administration (FDA) (Glenn and O'Hagan 2007; Uibo and Lernmark 2008). No consensus but several possible mechanisms has been proposed to explain how alum increases humoral immunity (Brewer *et al.* 1999; Jordan *et al.* 2004; Gavin *et al.* 2006a):

- Formation of a depot from which antigen is slowly released to enhance antibody production.
- Conversion of soluble antigen into a particulate form, enhancing phagocytosis by APCs such as macrophages, dendritic cells and B cells (Mannhalter *et al.* 1985).
- Induction of inflammation, with recruitment and activation of APCs that capture the antigen.
- Activation of dendritic cells by inducing production of the endogenous danger signal, uric acid (Kool *et al.* 2008).
- Activation of the NALP3/inflammasome complex, which is important in production of pro-inflammatory cytokines, for example IL-1 $\beta$  (Tritto *et al.* 2009).

### *Early clinical trials with GAD<sub>65</sub>*

In a double blind Phase I clinical study, 16 healthy male Caucasian volunteers, without HLA risk genotypes, received a single subcutaneous injection of unformulated rhGAD<sub>65</sub> and eight individuals received placebo (Diamyd Medical, unpublished). The study aimed to assess the safety and tolerability of increasing dose levels of rhGAD<sub>65</sub>, from 20 to 500  $\mu$ g. No adverse effects were observed at any dose level, and higher titres of GADA, IAA or IA-2A were not induced in any subject. From these results, GAD-alum treatment was considered to be clinically safe, and Phase II clinical studies in patients could therefore be conducted.

In the Phase IIa trial, subcutaneous injection of GAD-alum was given to patients diagnosed with Latent Autoimmune Diabetes in Adults (LADA). The disease process in LADA patients

resembles that in T1D in that they share genetic HLA susceptibility and T1D associated autoantibodies, especially GADA (Tuomi *et al.* 1999; Agardh *et al.* 2005). However, in T1D, the endogenous insulin secretion is lower and the rate of progression to insulin dependency is higher (Turner *et al.* 1997; Tuomi *et al.* 1999). The purpose of the study was to assess the clinical safety and efficacy, as well as the immunological impact of the antigen-based treatment in autoimmune diabetes. GAD-alum was tested in a dose-finding setup in 47 LADA patients. The lowest dose (4 µg) was not expected to give effect (Agardh *et al.* 2005). The 20 µg dose was considered appropriate for a highly immunogenic protein, while the 100 µg represented a commonly used vaccine dose level appropriate for moderately immunogenic proteins (Uibo and Lernmark 2008). The highest dose (500 µg) represented a dose level required for a poorly immunogenic protein. In addition to the four groups of patients receiving different doses of GAD-alum, one group received placebo (Agardh *et al.* 2005).

Clinical safety was reported from the Phase IIa study. Interestingly, it was shown that one of the doses (20 µg) increased fasting as well as stimulated C-peptide levels. The same concentration of GAD-alum induced a higher ratio of CD4<sup>+</sup>CD25<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cells, compared with the placebo group, suggesting a potential role of Treg cells in the immune modulation. Serum levels of IL-1β, -2, -4, -6, -8, -10, -12, GM-CSF and TNF were not influenced by the treatment (Agardh *et al.* 2005). In addition, only in the group that received the highest dose (500 µg), GADA levels were increased.

Since GAD-alum was reported to be safe and improve residual insulin secretion, a Phase IIb trial in T1D children diagnosed within the last 18 months was conducted by our group. Briefly, 70 T1D children positive for GADA and with fasting C-peptide above 0.1 pmol/mL were randomised to 20 µg GAD-alum or placebo (alum alone) at two occasions, one month apart. The clinical data showed a better preservation of fasting and stimulated C-peptide in the individuals with less than six months duration of disease at inclusion, still 30 months after intervention (Ludvigsson *et al.* 2008). The immunological findings at the 15 month follow-up showed that GAD<sub>65</sub> induced a broad range of cytokines but also expression of FOXP3 and TGF-β. Additional immunological findings from this study are described in paper III and IV and in the results and discussion of the thesis.

At present, phase III trials are underway both in Europe and in the USA (<http://clinicaltrials.gov/ct2/results?term=diamyd>). The European study is conducted in nine

countries (Sweden, the Netherlands, Finland, France, Germany, Italy, the UK, Slovenia and Spain). The inclusion has ended and the study comprises 334 T1D patients (10-20 years of age) diagnosed within the last three months, with fasting C-peptide levels above 0.1 pmol/mL and presence of GADA (Diamyd Medical AB, [www.diamyd.com](http://www.diamyd.com)). In this three-armed trial, one group will receive four subcutaneous injections with placebo; the second will be given 20 µg GAD-alum at two occasions and placebo at the other two visits; the third group will receive four injections of GAD-alum (<http://clinicaltrials.gov/ct2/results?term=diamyd>). Results from the European study will be available in the spring or summer of 2011 ([www.diamyd.com](http://www.diamyd.com)). The American study has a similar study protocol and is currently recruiting patients. Combination therapy with GAD-alum and β-cell stimulatory agents is also being tested in a clinical trial (<http://clinicaltrials.gov/ct2/results?term=diamyd>). It is unclear whether spontaneous β-cell regeneration will occur after immune modulation and if this could be induced by pharmacologic approaches. Glucagon-like peptide-1 (GLP-1) receptor agonists may augment insulin content of the recovered β-cells in type 2 diabetic (T2D) patients (Wajchenberg 2007) and has also shown positive effects in T1D (Behme *et al.* 2003; Dupre 2005).

In another trial, pancreatic biopsies will be obtained from adult high-risk individuals and newly diagnosed T1D patients, randomised to GAD-alum treatment or placebo (Ludvigsson 2009, oral communication). Prevention studies using GAD-alum in individuals at increased risk of developing T1D are underway, both in Europe and in the USA. A pilot study has started in Skåne, Sweden, recruiting children with increased risk of T1D from the DiPiS (Diabetes Prediction Study in Skåne) study, with the main aim to assess safety of GAD-alum treatment. The larger NorDiaPrev trial that will be conducted in Norway, Finland and Sweden aims to study both safety and efficacy. Ethical approval has recently been received. These studies will collectively add to the knowledge and help to uncover the mechanisms by which GAD<sub>65</sub> can delay or might even prevent the onset of autoreactivity.

## Aims of the thesis

The general aim of this thesis was to investigate the immunological profile of individuals with high risk of developing T1D and of children diagnosed with T1D and how this profile is influenced by immune intervention. The specific aims were:

- I. To study if the immune profile differs in high-risk individuals who did or did not develop manifest T1D and if Nicotinamide affects the immune balance.
- II. To study the chemokine and chemokine receptor profile in CD4<sup>+</sup> and CD8<sup>+</sup> cells from T1D children during the first 18 months after diagnosis.
- III. To study the immunomodulatory effect of GAD-alum shortly after treatment, with focus on cytokine secretion and expression of markers associated with regulatory cells.
- IV. To study the immunomodulatory effect of GAD-alum treatment, with focus on CD4<sup>+</sup>CD25<sup>high</sup> cells and their association with cytokine secretion.

## Material and methods

### Study populations

In this thesis, three different study populations were included: individuals with high risk of T1D participating in the ENDIT study, T1D children participating in the GAD<sub>65</sub>-vaccination study and healthy school children. An overview of the study populations in the thesis is shown in Table I.

*Table I. Study populations in the thesis.*

<b>Subjects</b>	<b>Paper</b>	<b>Number of subjects</b>	<b>Mean age (range)</b>	<b>Gender male/female</b>
High-risk first-degree relatives of T1D patients participating in the ENDIT study	I	12	(8-45)	6/6
• Developed T1D		6	20.3 (8-42)	3/3
• Non-diabetic		6	21.6 (9-45)	3/3
Children with T1D participating in the GAD <sub>65</sub> -vaccination trial, before the first injection	II	58	14.1 (10-18)	22/36
• 0-4 months duration		11	12 (11-18)	5/6
• 5-9 months duration		17	15 (12-18)	6/11
• 10-14 months duration		19	14 (10-18)	6/13
• 15-18 months duration		11	14 (12-16)	5/6
Children with T1D participating in the GAD <sub>65</sub> -vaccination trial	III, IV	70	14.2 (10-18)	28/42
Healthy school children	II, IV	12	13.5 (11-15)	4/8

## **European Nicotinamide Diabetes Intervention Trial (ENDIT)**

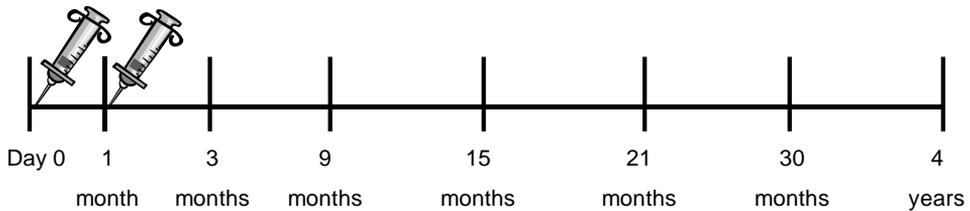
The ENDIT study included first-degree relatives of patients with T1D having increased risk of development of disease. More than 2000 first-degree relatives were screened in Sweden in order to identify individuals with a risk as high as 40% of developing the disease within five years ( $\geq 20$  ICA IJDF units). The individuals were randomised to either oral Nicotinamide (Ferrosan AC, Copenhagen, Denmark) at a dose of 1.2 g/m<sup>2</sup> daily, up to a maximum of 3 g/day, or placebo, for five years. Twenty-three high-risk first-degree relatives were included in Sweden, and during the trial, seven of them developed T1D. Blood samples from all participants were collected 2–5 years before (sample 1, S1), approximately 1 year before (S2) and close to the onset of T1D (S3). During the same period, blood samples (S1–S3) were collected from high-risk individuals, matched by gender and age that did not develop T1D during the ENDIT trial. Venous blood samples were collected and transported to Linköping within 24 hours.

## **GAD<sub>65</sub>-vaccination study**

The GAD<sub>65</sub>-vaccination study is a phase IIb, double-blind, placebo-controlled, multi-centre (Linköping, Stockholm, Göteborg, Halmstad, Malmö, Örebro, Jönköping and Borås) trial, which started in 2005. The aim was to investigate the impact of GAD-alum (Diamyd™, Diamyd Medical AB, Stockholm, Sweden; rhGAD<sub>65</sub> in alum formulation) in patients with newly diagnosed T1D. The study included 70 children (42 female, 28 male) between 10 and 18 years of age (median 14 years), diagnosed with T1D within the last 18 months. All patients had a fasting serum C-peptide level above 0.1 pmol/mL and presence of GADA, at inclusion. Exclusion criteria were for example T2D, treatment with immunosuppressants and presence of another serious disease or condition. Eligible patients were randomly assigned to 20 µg of GAD-alum (n=35) or placebo (alum alone; n=35) administered subcutaneously at two occasions, four weeks apart. Patients remained in the clinic for observation during three hours after injection.

The Pharmacy at the University Hospital MAS in Malmö, Sweden, packed the ampoules in per-patient boxes with each box containing two identical ampoules of either GAD-alum or placebo. The boxes were labelled with treatment numbers, from 1 up to 70, according to a computer generated randomisation list.

Venous blood samples were collected at baseline and at months 1, 3, 9, 15, 21 and 30, during the morning hours to avoid time of day differences and were transported to Linköping within 24 hours (Figure 9). To evaluate the long-term effect and safety of GAD-alum treatment, samples from the Phase IIb trial have been collected in a four year follow-up during the spring and summer of 2009. Laboratory analyses and interpretation of the results are underway.



*Figure 9. Overview of sample collections in the Phase IIb GAD<sub>65</sub>-vaccination trial.*

### **Healthy school children**

Healthy children were recruited from Björnkärsskolan and Rydsskolan in Linköping, Sweden. The children were asked to fill out a questionnaire together with their parents regarding presence of T1D, T2D, celiac disease, rheumatoid arthritis, goitre and allergies in first degree relatives and themselves. Children with any of the conditions above, or having first degree relatives with any of the conditions, were excluded. In paper II and IV, samples from 12 healthy children were used.

## Laboratory analyses

*Table II. Laboratory methods used in the thesis.*

Method	Paper
Isolation of peripheral blood mononuclear cells	I, II, III, IV
Cell culture	I, II, III, IV
Cytokine analysis	
- ELISPOT	I
- ELISA for CCL21	II
- Multiplex Luminex technique	II, III, IV
Flow cytometry	II, IV
Real-time RT-PCR analysis	III, IV
Detection of antibodies	
- ICA	I
- Radioimmunoassay for GADA	I, III, IV
Metabolic parameters	
- C-peptide	I, II
- Proinsulin	I

### Isolation of peripheral blood mononuclear cells

In all papers included in this thesis, peripheral blood mononuclear cells (PBMC) were isolated from sodium-heparinised venous blood samples using Ficoll Paque (Pharmacia Biotech, Sollentuna, Sweden) density gradient centrifugation for 30 min at 400 g. The mononuclear cells were collected from the interface of blood and Ficoll, whereas the erythrocytes sedimented to the bottom. Thereafter, the PBMC were washed twice by centrifugation for 15 min at 400 g in RPMI 1640 medium (Gibco, Täby, Sweden), supplemented with 2% fetal calf serum (FCS) (Gibco). The cells were diluted (1:10) in Türks solution and counted by light microscopy. After a final wash, the cells intended for subsequent cell culture (paper II and IV) were resuspended in AIM V research-grade serum-free medium (Gibco) supplemented with 2mM L-Glutamine, 50 µg/L Streptomycin Sulphate, 10 µg/L Gentamicin Sulphate and 20 µM

$\beta$ -mercaptoethanol (Sigma, Stockholm, Sweden), to a final concentration of  $1 \times 10^6$  PBMC/mL.

In paper I and III, the isolated PBMC were cryopreserved in liquid nitrogen after the last wash, awaiting further analyses. The cells were thawed in a  $+37^\circ\text{C}$  water bath under continuous agitation. After washing in RPMI 1640 supplemented with 10% FCS, the cells were resuspended in AIM V research grade supplemented with  $20 \mu\text{M}$   $\beta$ -mercaptoethanol. Cell viability was determined by trypan blue exclusion.

### **Stimulation of peripheral blood mononuclear cells**

In paper II,  $1 \times 10^6$  PBMC were cultured in AIM V medium (Gibco) supplemented with  $\beta$ -mercaptoethanol for 72 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ .

In paper III, additional cultures with  $5 \mu\text{g/mL}$   $\text{GAD}_{65}$  (Diamyd Medical, Stockholm, Sweden),  $5 \mu\text{g/mL}$  phytohemagglutinin (PHA) (Sigma),  $10 \mu\text{g/mL}$  IA-2 peptide,  $0.5 \mu\text{g/mL}$  insulin peptide,  $5 \mu\text{g/mL}$   $\text{GAD}$ -alum or placebo (Diamyd Medical) were performed under the same conditions as described above.

In paper IV, PBMC were cultured under the same conditions, exclusively with  $\text{GAD}_{65}$  or in medium alone. Cell supernatants were collected after 72 hours culture and frozen at  $-70^\circ\text{C}$  until analysed. The PBMC were resuspended in  $300 \mu\text{L}$  RLT lysis buffer (RNeasy 96 RNA extraction kit, Qiagen Sciences, Germantown, MD, USA) and frozen at  $-70^\circ\text{C}$  until used for real-time RT-PCR (reverse transcriptase - polymerase chain reaction).

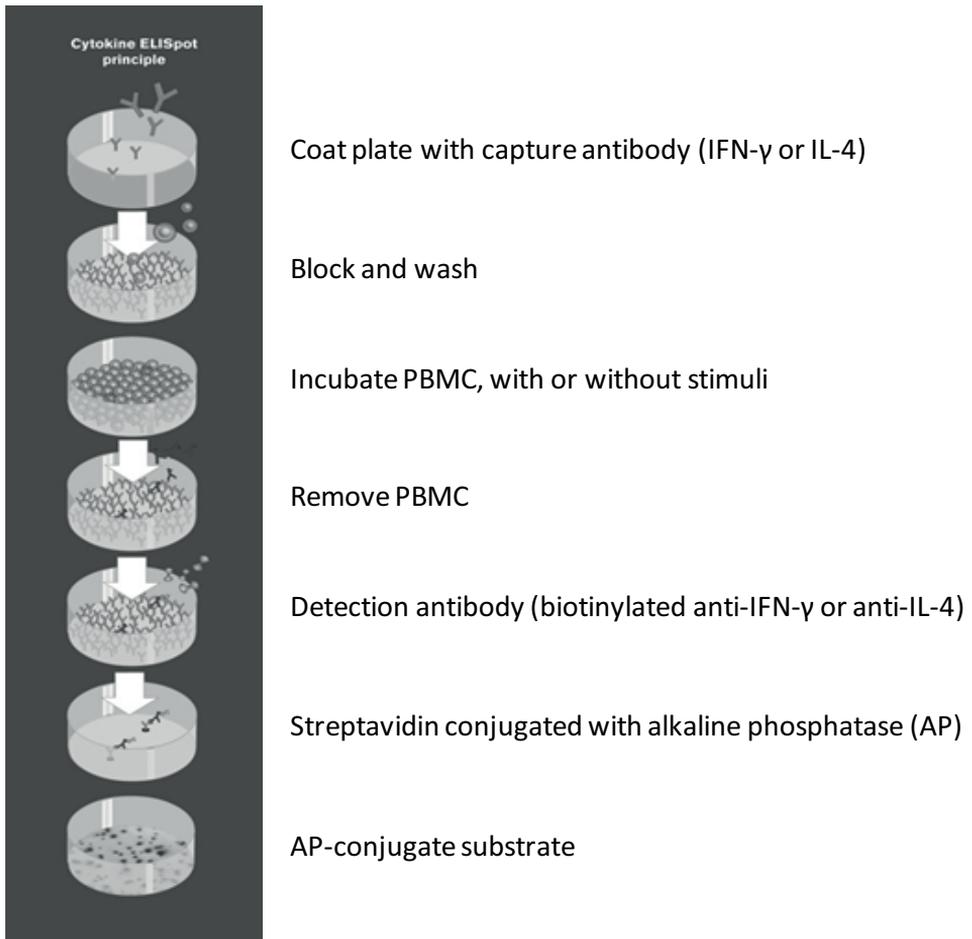
In paper IV, PBMC were cultured overnight (18 hours) with  $\text{GAD}_{65}$  or in culture medium alone, but otherwise treated as above for subsequent flow cytometric analyses.

### **Enzyme Linked Immunospot (ELISPOT)**

The enzyme linked immunospot (ELISPOT) assay detects secreted cytokines in low frequencies of cells (Kalyuzhny 2005). The benefit with ELISPOT is that the secreted cytokine is immediately captured by a coating antibody and can therefore not be digested or internalised by the cells. The ELISPOT procedure is illustrated in Figure 10.

Sterile PVDF 96-well microtitre plate (Multiscreen HA, Millipore, Bedford, Mass., USA) was treated with 70% ethanol and was thereafter washed five times with water to remove remaining alcohol. The plate was coated with mouse anti-human IFN- $\gamma$  monoclonal antibody (mAB) or mouse anti-human IL-4 mAB (Mabtech AB, Stockholm, Sweden) overnight at 4°C. Non-specific binding sites on the plate membrane were blocked by incubation with Iscove's modification of Dulbeccos medium (IMDM) (Life Technologies, Täby, Sweden) supplemented with 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin (Life Technologies), 10 ml/L 100 x MEM non-essential amino acids (Life Technologies) and 5% FCS, for 30 min at 37°C. The plate was washed with sterile PBS and thereafter IMDM was added to the plate. The medium was removed prior to addition of PBMC to the plate. Aliquots of  $1 \times 10^5$  PBMC/well were applied together with 100  $\mu$ L of IMDM with supplements, including one of the following antigens: GAD<sub>65</sub> (Diamyd Medical AB, Stockholm, Sweden), the synthetic peptide of GAD<sub>65</sub> (a.a. 247–279, Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden), IA-2 (produced in *Escherichia coli*, Åbo Akademi University, Turku, Finland), all at a concentration of 100 pg/mL; 10  $\mu$ g/mL HSP (DiaPep277, a.a. 437–460, The Weizmann Institute of Science, Rehovot, Israel) and 20  $\mu$ g/mL PHA (Sigma, Stockholm, Sweden). In samples with a limited number of cells, the order of priority for stimulation was PHA, GAD<sub>65</sub>, HSP peptide, IA-2 and GAD<sub>65</sub> peptide. All variants of differently stimulated and non-stimulated cells were applied in quadruplicate. As a control, some wells on each plate were incubated exclusively with culture medium without cells but were otherwise treated as the other wells. Stimulation with PHA was used as a positive control.

Cells were cultured undisturbed for 48 hours at 37°C, in a humidified atmosphere with 5% CO<sub>2</sub>. The plate was washed twice with phosphate buffered saline (PBS) and twice with PBS Tween. Thereafter, biotinylated anti-human IFN- $\gamma$  mAB and biotinylated anti-human IL-4 mAB was added and the plate was incubated for two hours at room temperature (RT). After four washes with PBS, streptavidin conjugated with alkaline phosphatase (AP) (diluted 1:1000) was added and the plate was incubated for one hour. The plate was washed again, twice with PBS Tween and four times with PBS. Spots were developed by AP conjugate substrate kit (Bio-Rad, Hercules, CA, USA). The reaction was allowed to proceed for 10-15 minutes before the wells were rinsed with excessive amounts of tap water, emptied and dried overnight at room temperature.



**Figure 10. Principle of the Enzyme Linked Immunospot (ELISPOT) assay technique.** (Mabtech AB, Sweden, 2009)

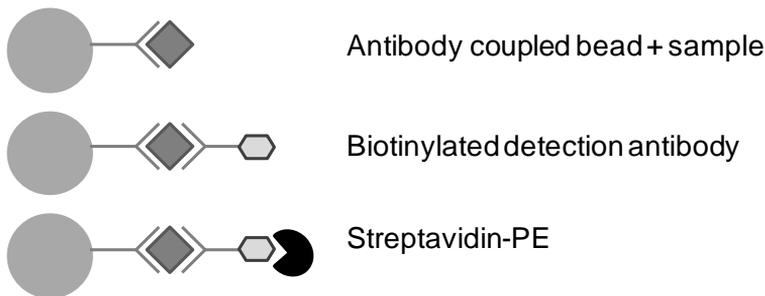
The ELISPOT plates were blinded for identity to avoid any influence on the outcome. The plates were counted automatically, by the AID ELISPOT Reader System (AID, Strasbourg, France). Each well was photographed and a software program marked the spots based on size, colour and intensity. However, manual supervision of each plate was required since artefacts sometimes could be incorrectly registered as spots. The median value of spots in the quadruplicates of wells was calculated for each antigen. Our laboratory participated in the first ELISPOT workshop as one of the core laboratories, and our assay was judged to be sensitive and reproducible (Schloot *et al.* 2003).

## Enzyme Linked Immunosorbent Assay (ELISA)

Secretion of the chemokine 6Ckine in cell supernatants was analysed with Quantikine human CCL21/6Ckine ELISA according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). Briefly, 100  $\mu$ L of assay diluent, sample, standard and blank were added to a 96-well microtitre ELISA plate. Samples were run in single wells (standards and blank in duplicates) and the plate was incubated for 2 hours at RT on a horizontal orbital shaker. The wells were then washed four times and 200  $\mu$ L of anti-6Ckine conjugate was added. The plate was incubated for another 2 hours and thereafter washed 4 times before adding 200  $\mu$ L substrate solution. The plate was incubated for 20 minutes at RT, protected from light. Fifty  $\mu$ L of stop solution was added to each well and the optical density was determined within 30 minutes with a microplate reader set to 450 nanometres. The sensitivity for the analysis of 6Ckine by ELISA was 13.1 pg/mL.

## Multiplex fluorochrome technique (Luminex)

Cytokines and chemokines were analysed by multiplex fluorochrome technique. In paper II, secretion of the chemokines IP-10, MCP-1, MIP-1 $\alpha$ , -1 $\beta$  and RANTES were analysed in cell supernatants. In papers III and IV, the cytokines IL-5, -6, -10, -12 (p70), -13, -17, TNF and IFN- $\gamma$  were measured in cell supernatants and in serum samples. The Bio-Plex™ Cytokine Reagent Kit (Bio-Rad Laboratories) was employed in all assays with cell supernatants, and human serum diluent kit was used for the analyses of serum samples. The Luminex technique is a sandwich immunoassay based on colour coded microspheres, to which antibodies directed against the cytokine or chemokine of interest is covalently coupled (Figure 11). The method allows simultaneous detection of up to 100 different molecules in a small sample volume.



*Figure 11. Principle of the multiplex fluorochrome technique (Luminex).*

Sample, standard, blank and colour-coded beads were added to a 96-well microtitre plate. Samples were run in single wells (standards and blank in duplicates). After incubation for 30 minutes at RT and washing with a vacuum device, biotinylated detection antibody mixture was added and the plate was incubated for another 30 minutes at RT. After washing, the plate was incubated for 10 minutes with streptavidin-PE (phycoerythrin) and was thereafter washed again. The beads were resuspended in assay buffer before analysing the samples. Identification and quantification was performed with a Luminex100™ instrument (Luminex xMAP™ Technology, Austin, TX, USA), based on bead colour and fluorescence. The blank responses were subtracted from the response from the samples. Acquisition conditions were set with a minimum of 100 beads per region. Median fluorescence intensity (MFI) was analysed using Starstation Software version 2.3 (Applied Cytometry Systems, Sheffield, UK) and a five-parameter curve fit was applied to each standard curve in order to calculate sample concentrations. The cut-offs for minimum detectable concentration (pg/mL) for each chemokine and cytokine were as follows: 15.04 (IP-10); 1.66 (MCP-1); 0.32 (MIP-1 $\alpha$ ); 0.47 (MIP-1 $\beta$ ); 0.76 (RANTES); 0.5 (IL-5, IL-6, IL-13), 0.6 (IL-10, IL-12, IFN- $\gamma$ ), 0.7 (IL-17) and 1.3 (TNF).

## **Flow cytometry**

Flow cytometry was employed in paper II and IV. In paper II, the extracellular expression of chemokine and cytokine receptors was analysed on freshly isolated PBMC. The PBMC were washed in PBS (Medicago AB, Uppsala, Sweden) containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) by centrifugation at 500 g during 10 minutes. The cells were incubated for 30 minutes at 4°C in darkness with: fluorescein isothiocyanate (FITC) conjugated mouse anti-human CXCR3, CCR7 (R&D Systems, Minneapolis, MN, USA), CCR5 (PharMingen, San Diego, CA, USA), CD45RA and CCR8 (BD Biosciences, San Jose, CA, USA); PE conjugated anti-IL-18R, CXCR6 (R&D Systems), IL-12R $\beta$ 2, CCR4 (PharMingen) and CD45RO (BD Biosciences); peridinin chlorophyll (PerCP) conjugated anti-CD8 and allophycocyanin (APC) conjugated anti-CD4 (BD Biosciences). After incubation, the cells were washed as before and resuspended in PBS + 0.1% BSA and kept at 4°C in darkness until analysed. Unstained PBMC and isotype controls were included to estimate the amount of autofluorescence and non-specific binding. Cells stained with single antibodies marked with different fluorochromes were used for compensation to adjust for spectrally adjacent dye pairs. Four-colour flow cytometry was performed with a Becton Dickinson fluorescence activated cell sorter (FACSCalibur) (San Diego, CA, USA).

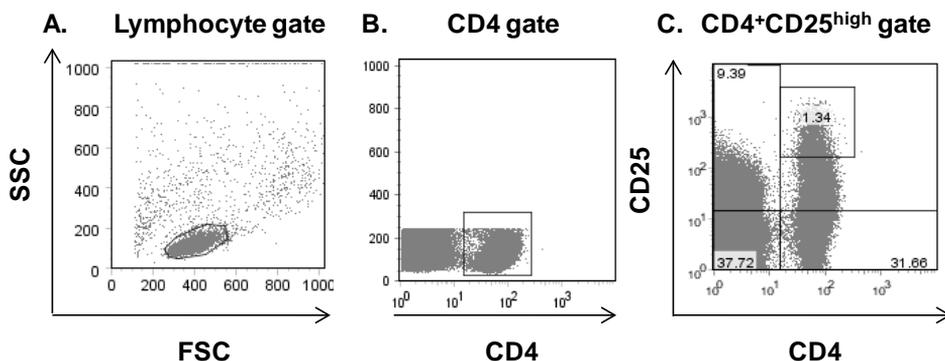
Approximately  $1 \times 10^4$  lymphocytes were acquired for unstained lymphocytes, isotype and compensation, while  $2 \times 10^4$  lymphocytes were acquired for analysis of cell surface receptors.

In paper IV, the extra- and intracellular expression of markers associated with regulatory cells were studied in freshly isolated PBMC from baseline and from the 3, 9 and 15 month follow-ups. At the 21 and 30 month visits, samples were cultured overnight in medium alone or with addition of  $5 \mu\text{g/mL}$  GAD<sub>65</sub> at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  before flow cytometry analyses. Samples collected at baseline and at 3 and 9 months were stained with CTLA-4, Neuropilin-1, CD4 and CD25. Neuropilin-1 was chosen after a report suggesting its expression as a suitable surface marker for Treg identification and discrimination from recently activated T cells (Bruder *et al.* 2004). Scarce expression of TGF- $\beta$ 1 (Anti-human LAP) (R&D Systems) has been observed by us on several occasions, and therefore TGF- $\beta$ 1 was excluded from our experiments. Extracellular staining of  $2 \times 10^6$  PBMC was performed with CD4 PerCP (Pharmingen, San Diego, CA, USA) and CD25 APC (BD Biosciences, San Jose, CA, USA), primary polyclonal anti-human anti-Neuropilin-1 (Santa Cruz Biotechnology, CA, USA) and FITC-conjugated goat IgG (R&D Systems, MN, USA). For intracellular staining of CTLA-4, the PBMC were incubated with 4% paraformaldehyde (Merck, Germany) and thereafter with Perm2 permeabilizing buffer (BD Biosciences). After washing, CTLA-4 PE (Pharmingen) was added and the cells were washed and left at  $4^\circ\text{C}$  in darkness until flow cytometry analysis.

The FOXP3 antibody was not available at the beginning of the trial, but was included in the analyses at 15, 21 and 30 months. The FOXP3 antibody clone PCH101 (eBioscience, San Diego, CA, USA) has been shown to recognise both isoforms of the FOXP3 protein (Allan *et al.* 2007). For intracellular staining of FOXP3 and CTLA-4, the PBMC were treated with Fixation/Permeabilization buffer (eBioscience) for 30 minutes at  $4^\circ\text{C}$  in darkness after extracellular staining. The cells were washed and resuspended in Permeabilization buffer (eBioscience) and thereafter stained with anti-CTLA-4 and FOXP3 as previously described, and kept at  $4^\circ\text{C}$  in darkness until analysis. Approximately  $1 \times 10^4$  lymphocytes were acquired for autofluorescence, isotype, and compensation, while  $2 \times 10^5$  to  $6 \times 10^5$  lymphocytes were collected for analysis of Treg associated markers.

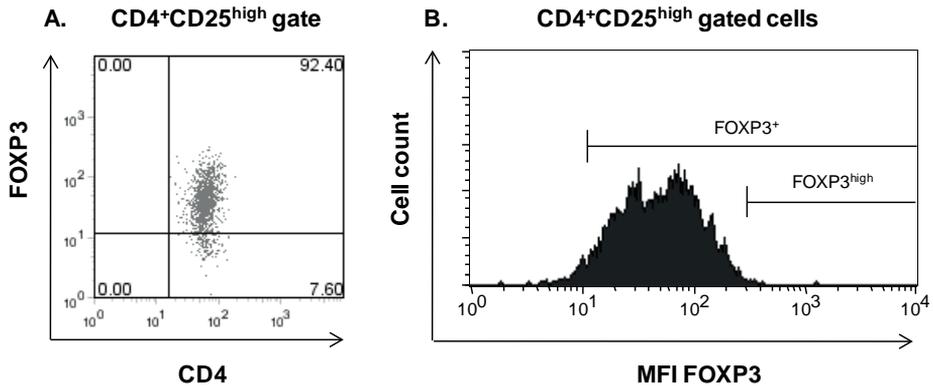
### Strategy for gating and analysis of flow cytometric data

The data was analysed using the CellQuest program (Becton Dickinson Immunocytometry Systems). All analyses were performed in a blinded manner. Lymphocytes were gated by forward (FSC) and side scatter (SSC) (Figure 12 A). CD4<sup>+</sup> cells were gated according to positivity for CD4 as defined by the isotype controls (Figure 12 B). The CD25<sup>high</sup> gate was adjusted to contain CD4<sup>+</sup> cells that expressed higher levels of CD25 than the discrete population of CD4<sup>+</sup>, for each individual sample (Figure 12 C) (Hoffmann *et al.* 2004; Mjosberg *et al.* 2009).



**Figure 12. Gating strategies for flow cytometry analyses.** (A) Gating of lymphocytes was adjusted according to forward scatter (FSC) and side scatter (SSC) and (B) gating of CD4<sup>+</sup> cells was determined according to CD4 positivity. (C) The CD4<sup>+</sup>CD25<sup>high</sup> gate was adjusted to contain CD4<sup>+</sup> cells that expressed higher levels of CD25 than the discrete population of CD4<sup>+</sup> cells.

Results are expressed as the percentage of cells expressing each molecule, or as MFI, which is proportional to the amount of molecules present on the cells. Representative percentage and MFI of FOXP3 in CD4<sup>+</sup>CD25<sup>high</sup> cells is shown in Figure 13 A-B. The MFI of CD25 in CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup> gated cells were analysed in cells above the CD25<sup>high</sup> level. To avoid errors when subjectively setting gates, the 0.2% expressing the highest levels of CD25, FOXP3, CTLA-4 and Neuropilin-1 were evaluated in CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup> gated cells.



**Figure 13. Representative plots from flow cytometry analyses. (A) Representative expression of FOXP3 in CD4<sup>+</sup>CD25<sup>high</sup> gated cells. (B) Representative median fluorescence intensity (MFI) of FOXP3 in CD4<sup>+</sup>CD25<sup>high</sup> gated cells.**

## Real-time RT-PCR

### RNA isolation and cDNA synthesis

In paper III and IV, total RNA was isolated from lysed PBMC according to the RNeasy 96 vacuum/spin protocol (Qiagen, Solna, Sweden). Cell lysates were thawed at room temperature and mixed with 70% ethanol (1:1) and then applied to an RNeasy 96-well plate. Contaminants were washed away by several centrifugation steps using wash buffers included in the kit. The RNA was eluted in RNase-free water and quantified by optical density (OD) measurements at 260 nanometres using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity of the RNA was ensured with an OD 260/280 ratio above 1.8.

Complementary DNA (cDNA) was synthesised from total RNA using the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). RNA, at a concentration of 7 ng/μL, was added to the reaction mix including MultiScribe reverse transcriptase, random primers, dNTPs, reverse transcription buffer, RNase inhibitor and RNase free water. Reverse transcription (RT) of total RNA to cDNA was performed with a GeneAmp® PCR system 2700 (Applied Biosystems). The reaction was run during 10 minutes at 25°C, followed by 2 hours at 37°C. The final cDNA product was stored at -20°C for subsequent cDNA amplification by real-time polymerase chain reaction (PCR).

### **Quantification of FOXP3 and TGF- $\beta$ mRNA with real-time PCR**

FAM-labelled primers/probes were used to determine transcription levels of FOXP3 (Hs00203958) or TGF- $\beta$  (Hs00171257) (Applied Biosystems), and VIC-labelled primers/probes were used for the endogenous control 18s rRNA (4310893E) (Applied Biosystems). The cDNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and was thereafter transferred to MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems) in duplicates. In all experiments, controls without template (no template control, NTC) as well as an inter assay control RNA (internal control, prepared from unstimulated PBMC of a healthy individual) were included. The reaction mixture was amplified using the 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal cycle conditions were: 50°C for 2 min followed by 95°C for 10 min and then 50 cycles were run with 15 sec at 95°C and 1 min at 60°C.

Gene transcription in the samples was quantified using a comparative cycle threshold (Ct) method, according to the manufacturer's description (Applied Biosystems user bulletin #2 PN 4303859). Briefly, a threshold line was set in the exponential phase of amplification, and the cycle at which the sample reaches this level was defined as Ct. The mRNA expression for each sample was calculated by subtracting the Ct value for the endogenous control 18s rRNA from the Ct value for the target mRNA, and expressed as  $\Delta$ Ct. A low Ct value represents high mRNA expression, and vice versa. Spontaneous mRNA expression was regarded as the  $\Delta$ Ct value from non-stimulated samples. To calculate the autoantigen induced mRNA expression, the  $\Delta$ Ct value for spontaneous expression was subtracted from the stimulated sample's  $\Delta$ Ct value to obtain the  $\Delta\Delta$ Ct value. The autoantigen-induced mRNA expression was then defined as relative transcription, based on the calculation  $2^{-\Delta\Delta$ Ct}.

### **Islet cell antibodies (ICA)**

Islet cell antibodies were detected with immunofluorescence on human pancreas sections according to a previously reported method (Bottazzo *et al.* 1974). A FITC-conjugated anti-IgG was used for detection (Bingley *et al.* 1997).

### **Glutamic acid decarboxylase antibodies (GADA)**

Serum GADA titres were determined using a radio binding assay employing  $^{35}$ S-labelled recombinant human GAD<sub>65</sub> produced by *in vitro* transcription/translation (pEx9 vector kindly

supplied by Prof. Åke Lernmark, University of Washington, Seattle, WA, USA). Sepharose protein A was used to separate free from antibody bound labelled GAD<sub>65</sub>.

A standard curve, consisting of two fold dilutions of a sample from one individual positive for GADA, was included on each plate. Wells containing only buffer were included as blanks. Positive and negative controls were included in each plate. The immunoprecipitated radioactivity was counted on a Wallac 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences, Inc, Boston, MA, USA), and the results were expressed as RA U/mL of autoantibodies in relation to the standard curve. Cut-off for positivity was regarded as 67.3 RA U/mL (23.1 WHO units) based on the 95<sup>th</sup> percentile of measurements from 1700 children, age 5-6 years, participating in the ABIS (All Babies of Southeast Sweden) study. In the Diabetes Autoantibody Standardisation Program (DASP) workshop in 2005 (laboratory number 221), our assay had a 96% specificity and 76% sensitivity and the test was calculated for a 98<sup>th</sup> percentile cut-off. Inter assay variations for the negative and positive controls were 10% and 8%, respectively.

All samples in the GAD<sub>65</sub>-vaccination study were tested in four replicates, with duplicate wells in two plates simultaneously assayed. To exclude inter assay variation, samples from each patient collected before the first injection and after 1, 3, 9 and 15 months were run simultaneously. Samples collected at the 21 and 30 month controls were simultaneously analysed, in parallel with selected samples from the 15 month control that were included to minimise inter assay variations.

### **C-peptide & Proinsulin**

The levels of connecting peptide (C-peptide) and proinsulin, in paper I, were determined by ELISA technique (AH Diagnostics, Stockholm, Sweden). C-peptide levels in paper II were measured in serum samples with a Time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit, Wallac, Turku, Finland). Results were validated with inclusion of a C-peptide control module containing a high, a medium and a low-level control in each assay (Immulite, DPC, UK). A 1224 MultiCalc® program (Wallac) was used for automatic measurement and result calculation. Measurements were expressed in pmol/mL.

## **Statistical analyses**

The data was not normally distributed and non-parametric statistical analyses, corrected for ties, were used throughout the whole thesis. For comparison between three or more groups, the Kruskal-Wallis test was employed and two groups were compared by Mann-Whitney U-test. Spearman's rank order correlation test was used when correlating non-parametric variables. In paper III, differences within the GAD-alum and placebo groups were analysed by Friedman's test followed by Wilcoxon signed rank test. A probability level of  $<0.05$  was considered to be statistically significant, whereas  $p<0.1$  was regarded as a trend. Calculations in paper I were performed using the statistical package STATVIEW 5.0.1 for Macintosh (Abacus Concepts Inc., Berkeley, CA) and for paper II, III and IV SPSS for Windows (SPSS Inc., Chicago, IL, USA), version 12.0.1; 15.0 and 17.0 were used.

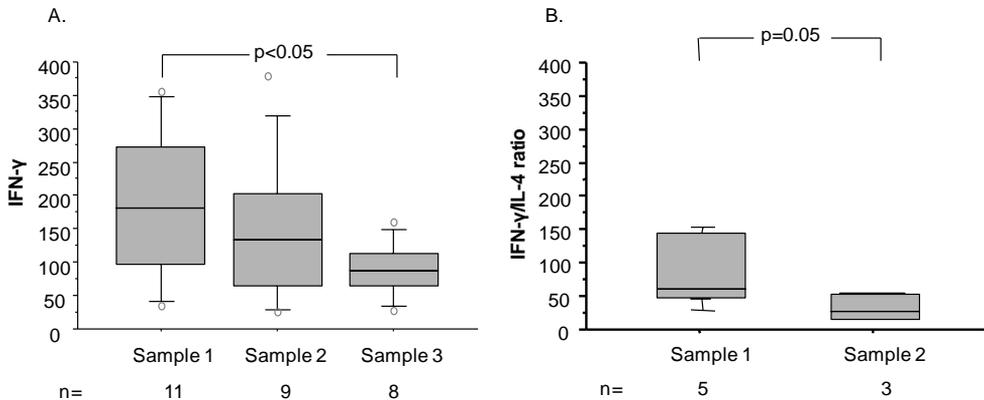
## **Ethical considerations**

Collection of blood samples included in the studies was performed after informed consent was obtained from the children and their parents or the participant if he/she was over 18 years of age. The studies were approved by the Regional Ethics Committee for Human Research at the Faculty of Health Sciences, Linköping University, Linköping, Sweden.

## Results & Discussion

### Th1 associated immune deviation in high-risk individuals

It has previously been shown that the Th1 like dominance in the pre-diabetic phase is diminished at diagnosis of the disease (Karlsson *et al.* 2000; Halminen *et al.* 2001; Karlsson Faresjo *et al.* 2004). This could be a consequence of reduced function of Tregs, which has been proposed as a potential predisposing factor of autoimmunity (Lindley *et al.* 2005). We have been able to confirm the dominant Th1 like profile, represented by high spontaneous secretion of IFN- $\gamma$ , in individuals at increased risk of developing T1D. Close to the onset of clinical T1D, the IFN- $\gamma$  secretion was decreased (Figure 14 A). Also the IFN- $\gamma$ /IL-4 ratio decreased in high-risk individuals who later developed T1D (Figure 14 B).



**Figure 14.** Cytokine secretion of IFN- $\gamma$  and IL-4 (spots/100 000 PBMC) was analysed in high-risk individuals 2–5 years before (Sample 1), approximately 1 year before (Sample 2) and close to the onset of T1D (Sample 3). During the same period (S1–S3), secretion of cytokines was analysed from high-risk individuals who did not develop T1D during the trial. (A) Spontaneous IFN- $\gamma$  secretion gradually decreased in high-risk individuals during the ENDIT trial. (B) A diminished ratio of spontaneous IFN- $\gamma$ /IL-4 was found in those who later developed T1D. The figure illustrates the number of cytokine-secreting cells/100 000 PBMCs, detected by ELISPOT technique, in box plots (10<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, and 90<sup>th</sup> percentile and outliers are indicated).

At time of inclusion in the ENDIT trial, IFN- $\gamma$  secretion was inversely correlated to the levels of ICA, in high-risk individuals who remained healthy. Induced IL-4 secretion in response to GAD<sub>65</sub> and IA-2 was found in the same individuals. These findings could suggest a Th2

deviated response in the high-risk individuals who remained healthy. In addition, the IA-2 induced IL-4 response was positively correlated to C-peptide in these individuals, which implies that Th2 response to autoantigens is associated with  $\beta$ -cell preservation.

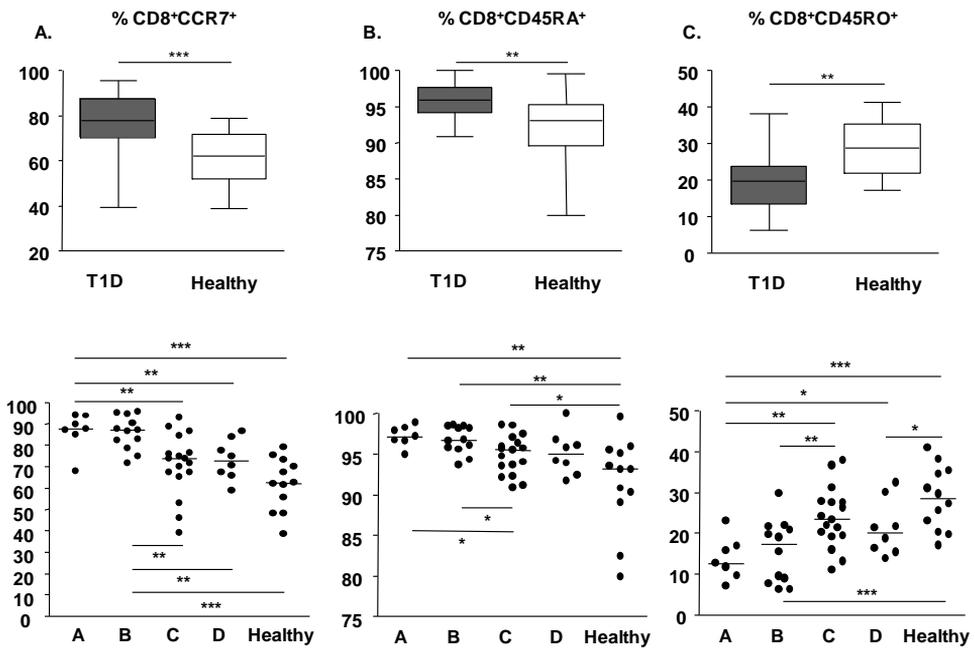
During 2009, the individuals at increased risk of T1D that remained healthy during the ENDIT trial were contacted. The purpose was to receive information about development of T1D. Out of the six healthy individuals in our study, four answered that they had not developed T1D. One of the participants had developed the disease during the last year and one did not answer. New statistical analyses, where the individual previously categorised as healthy instead was included in the T1D group, resulted in loss of significance for some of the parameters. This is likely to be explained in part by the low numbers of individuals in the groups, but also due to the fact that the person that developed T1D did so as late as ten years after the last sample was collected. The possibility that this individual had an active autoreactive process at the time is small, possibly explaining why the immunological profile does not correspond with the diabetic patients.

### **Altered CD8<sup>+</sup> cell phenotype in type 1 diabetic patients**

We have examined whether the chemokine and chemokine receptor expression changed during the period from diagnosis of T1D until 18 months later, as a lead in understanding how duration of disease might have an impact on efficacy of intervention. The expression of chemokine receptors, but also markers associated with memory and naïve cells were analysed on CD8<sup>+</sup> and CD4<sup>+</sup> cells by flow cytometry in samples from T1D children, recently included in the GAD<sub>65</sub>-vaccination study and in healthy children, included as reference. All samples from the T1D patients were collected prior to the first injection with GAD-alum or placebo and were grouped into four categories according to disease duration at the time of blood sampling.

Chemokines and chemokine receptors are important in directing leukocyte migration between blood, lymph nodes and tissues. They are key players in the migration of pathogenic T cells into the pancreatic islets of NOD mice developing T1D (Bradley *et al.* 1999), and presumably also in humans. The receptor expression changes as cells differentiate from naïve into effector cells. Expression of the CCR7 receptor defines naïve and T<sub>CM</sub> cells with ability to circulate repeatedly into lymphoid nodes (Worbs and Forster 2007). We found higher percentages of cytotoxic CD8<sup>+</sup> T cells expressing CCR7 but also CD45RA, defining naïve cells, in diabetic

compared with healthy children (Figure 15 A-B). This was especially evident in the group of patients with short disease duration, while in patients with a longer history of disease the percentages declined to levels similar to the healthy group. In contrast, the percentage of  $CD8^+CD45RO^+$  cells, defining antigen-experienced memory cells, was lower among diabetic individuals and increased with disease progression (Figure 15 C). Similar findings have been made by others in  $CD4^+$  cells, showing higher percentages of  $CD45RA^+$  cells in pre-diabetic twins, compared with twins who remained healthy and instead had elevated percentages of memory  $CD4^+CD45RO^+$  cells (Peakman *et al.* 1994).

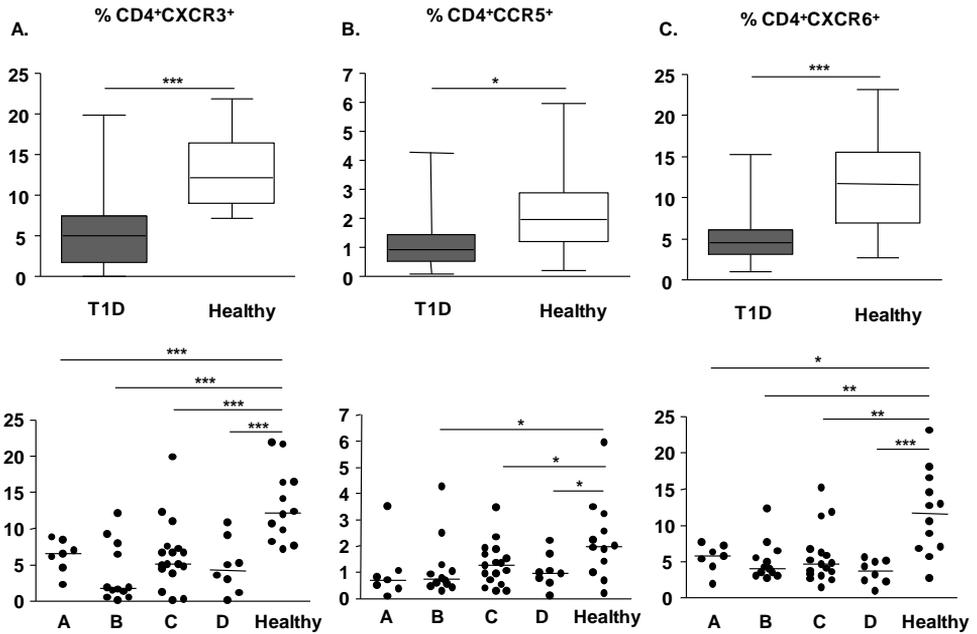


**Figure 15.** Expression of (A) CCR7, (B) CD45RA and (C) CD45RO on  $CD8^+$  cells determined by flow cytometry. The percentages (%) of cells expressing each receptor in the diabetic (T1D) ( $n=44$ ) and healthy ( $n=12$ ) group are shown in box-plots. Horizontal lines represent the median, the box comprises the 25<sup>th</sup> and 75<sup>th</sup> percentiles and the whiskers comprise remaining data. Expression of CCR7, CD45RA and CD45RO on  $CD8^+$  cells are shown in relation to duration of T1D in comparison with healthy controls. Patients were grouped according to duration of disease: 0–4 months (A,  $n=7$ ); 5–9 months (B,  $n=12$ ); 10–14 months (C,  $n=17$ ) and 15–18 months (D,  $n=8$ ); healthy ( $n=12$ ). Asterisks indicate the level of significance: \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

Naïve CD8<sup>+</sup> cells divide and differentiate into CD8<sup>+</sup> effector cells after antigen encounter. This is followed by a programmed contraction of the CD8<sup>+</sup> cells, leading to a smaller population of persisting antigen-specific CD8<sup>+</sup> memory cells (Hinrichs *et al.* 2006). These findings are consistent with our observations of decreasing levels of CD8<sup>+</sup> cells expressing CCR7 and CD45RA and increasing expression of memory CD45RO<sup>+</sup> Tc cells with duration of disease. The progressive destruction of the pancreatic  $\beta$ -cells in the course of T1D ultimately leads to a reduced amount of autoantigen and subsequently reduced local inflammation. Since autoantigens have a chemotactic effect on leucocytes to alert the immune system of tissue damage (Oppenheim *et al.* 2005), lower antigen presentation might lead to a reduction of T cells infiltrating the pancreatic area. It has been suggested that the peripheral CD8<sup>+</sup> T cell autoreactivity is suppressed when the majority of  $\beta$ -cells have been destroyed (Pinkse *et al.* 2005).

### **Reduced levels of Th1 cells and chemokine secretion in T1D**

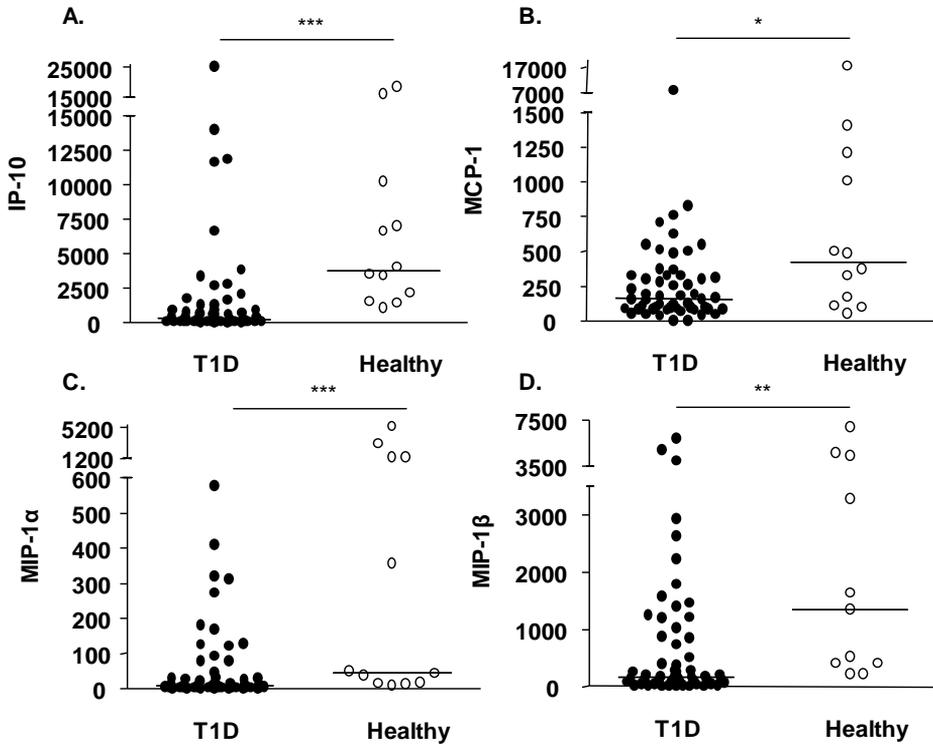
In parallel with increased percentages of CD8<sup>+</sup> cells with CCR7 and CD45RA phenotypes, a reduced expression of the Th1-associated chemokine receptors CCR5, CXCR3 and CXCR6 on CD4<sup>+</sup> cells (Figure 16 A-C) and a lower secretion of the chemokines IP-10, MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  in cell supernatants were detected in samples from T1D compared with healthy children. Reduced expression of CXCR3 and CCR5 on CD4<sup>+</sup> cells has previously been shown in T1D patients compared with healthy controls (Lohmann *et al.* 2002). T cells lose their expression of CXCR6 after antigen activation and recruitment into inflamed tissues (Koprak *et al.* 2003), which suggests that our findings of reduced CXCR6 levels in peripheral blood mirrors the recruitment of cells into the inflamed pancreas. A possible explanation for the reduction of CD4<sup>+</sup> cells expressing CCR5, CXCR3 and CXCR6 could be selective recruitment of Th1 cells into the pancreas. T cells infiltrating inflammatory sites are usually of the activated/memory phenotype (Qin *et al.* 1998). High proportions of memory CD45RO<sup>+</sup> T cells expressing CXCR3 and CCR5 have been observed in inflammatory lesions of patients with rheumatoid arthritis, while relatively low levels were detected in the peripheral blood or lymph nodes (Qin *et al.* 1998).



**Figure 16. Expression of (A) CXCR3, (B) CCR5 and (C) CXCR6 chemokine receptors on  $CD4^+$  cells determined by flow cytometry.** The percentages (%) of cells expressing each receptor in the diabetic (T1D) ( $n=44$ ) and healthy ( $n=12$ ) groups are shown in box-plots. Horizontal lines represent the median, the box comprises the 25<sup>th</sup> and 75<sup>th</sup> percentiles and the whiskers comprise remaining data. The percentages of CXCR3, CCR5 and CXCR6 on  $CD4^+$  cells are shown in relation to duration of T1D in comparison with healthy controls. Patients were grouped according to duration of disease: 0–4 months (A,  $n=7$ ); 5–9 months (B,  $n=12$ ); 10–14 months (C,  $n=17$ ) and 15–18 months (D,  $n=8$ ); healthy ( $n=12$ ). Asterisks indicate the level of significance: \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

Spontaneous secretion of IP-10, MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  in PBMC supernatant was lower in T1D patients compared with healthy children (Figure 17 A-D), which is in contrast to previous findings of elevated levels of serum IP-10 (Nicoletti *et al.* 2002; Rotondi *et al.* 2003). High secretion of MCP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  in PBMC supernatant has also been found in T1D patients compared with healthy individuals (Lohmann *et al.* 2002; Stechova *et al.* 2007). IP-10 accelerates the autoimmune process by enhancing the migration of antigen-specific lymphocytes to their target site (Rhode *et al.* 2005). We observed that secretion of IP-10 in T1D patients correlated with its receptor CXCR3, in contrast to CCR5 that did not correlate with any of its ligands MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES. Both in human and

experimental animal models of inflammation, deficiency of CCR5 was associated with increased levels of its ligand in tissue (Locati and Murphy 1999; Carr *et al.* 2006).



**Figure 17. Spontaneous secretion of the chemokines (A) induced protein (IP)-10, (B) monocyte chemoattractant protein (MCP)-1, (C) macrophage inflammatory protein (MIP)-1 $\alpha$  and (D) MIP-1 $\beta$  in cell culture supernatant from type 1 diabetic (T1D; black circles; n=54) and healthy children (white circles; n=12), determined by multiplex fluorochrome technique (Luminex). The chemokine concentrations are expressed in pg/mL. Horizontal lines indicate median values. Asterisks indicate the level of significance: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .**

The divergence in results from different studies might be explained by differences in study populations, detection methods and biological samples used. Spontaneous secretion in cell supernatant indicate the *in vitro* capacity of the PBMC to secrete chemokines without stimulation, while chemokine levels in serum might reflect the *in vivo* production at inflammatory sites by cells other than PBMC. The reduced amount of circulating Th1 like cells in T1D children, together with our previous observation of low secretion of IFN- $\gamma$  after diagnosis (Karlsson *et al.* 2000; Hedman *et al.* 2006), might be related to the low secretion of

the IFN- $\gamma$  inducible protein, IP-10, and possibly also to the reduced secretion of other chemokines such as MIP-1 $\alpha$ , MIP-1 $\beta$  and MCP-1.

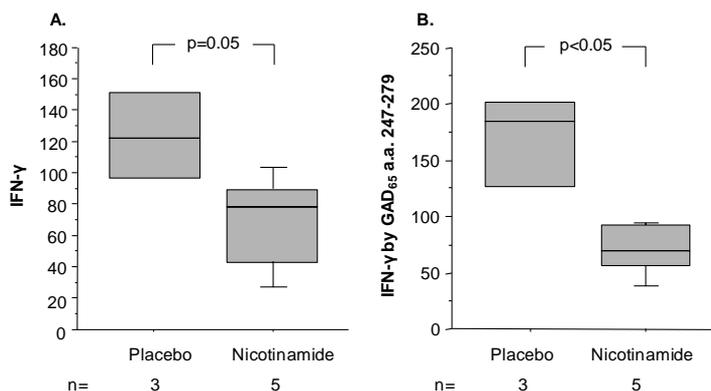
One of the main limitations in T1D studies in humans is the restriction to perform analyses in peripheral blood, leaving only hypotheses to understand the autoimmune inflammatory process at the site in the pancreas. Our findings of elevated expression of naïve CD8<sup>+</sup> Tc cells in parallel with reduced numbers of memory Tc and Th1 cells in the periphery of newly diagnosed T1D patients could be an effect of a selective recruitment of effector T cells to the pancreas, a decreased destructive process or an impaired T cell function in T1D patients.

## **Immunological effect of intervention**

### **Nicotinamide reduced high secretion of IFN- $\gamma$**

The ENDIT trial included high-risk first-degree relatives of T1D patients receiving either the B vitamin Nicotinamide or placebo. Preservation and regeneration of pancreatic  $\beta$ -cells in the pancreas were two proposed benefits of Nicotinamide treatment (Gale 1996). However, no clinical benefit was observed in the extensive study (Gale *et al.* 2004). Despite that, we have found indications of immunological effects of Nicotinamide in the small group of Swedish patients included in our study. High-risk individuals who received Nicotinamide during 4-5 years had significantly lower IFN- $\gamma$  secretion compared with the placebo group, both spontaneously (Figure 18 A) and after antigen stimulation (Figure 18 B). In addition, individuals receiving Nicotinamide tended to release less proinsulin in comparison with those receiving placebo, indicating a better  $\beta$ -cell function.

High levels of IFN- $\gamma$  and TNF in autoimmune diabetes induce apoptosis of pancreatic  $\beta$ -cells and induce the expression of MHC class II molecules. Nicotinamide has been shown to counteract the MHC class II expression on insulinoma cells from NOD mice and on human endothelial cells and fibroblasts, suggesting a potential protective mechanism of action by suppressing antigen presentation (Otsuka *et al.* 1991; Kim *et al.* 2002). In addition, the harmful effects of cytokines (IL-1 $\beta$ , IL-12, TNF, and IFN- $\gamma$ ) on human cultured pancreatic islets have been found to be inhibited by Nicotinamide (Eizirik *et al.* 1994; Kretowski *et al.* 2000).

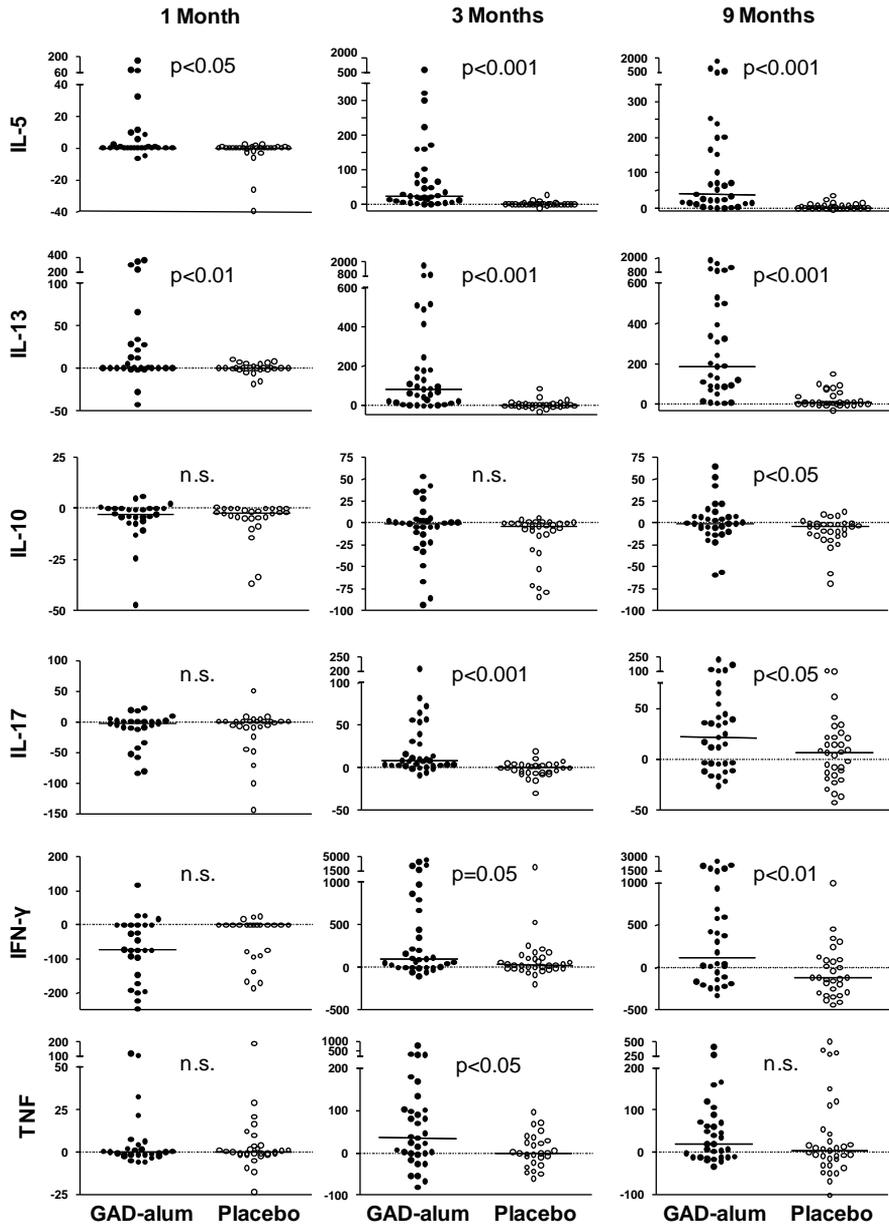


**Figure 18.** (A) Treatment with Nicotinamide during 4-5 years decreased the spontaneous IFN- $\gamma$  secretion (spots/100 000 PBMC), and (B) reduced IFN- $\gamma$  response against the GAD<sub>65</sub> peptide in high-risk individuals receiving Nicotinamide (2/5 developed T1D) compared with placebo (0/3 developed T1D).

Our results indicate that Nicotinamide may influence both  $\beta$ -cell function and the cytokine profile even though it appears not to protect against development of T1D, at the dosage used.

### GAD-alum induced an early Th2 response and a broader range of cytokines over time

We have previously shown clinical benefits and interesting immunological findings of GAD-alum treatment in T1D children, such as GAD<sub>65</sub>-induced cytokine secretion 15 months after intervention (Ludvigsson *et al.* 2008). In paper III, we wanted to further explore how the early cytokine secretion was affected by treatment with GAD-alum. Therefore, secretion of IL-5, -6, -10, -12 (p70), -13, -17, TNF, and IFN- $\gamma$  was analysed in PBMC supernatant after 72 hours *in vitro* culture. We hypothesised that a predominant Th2 protective immune response would follow the GAD-alum treatment in humans, based on the findings previously made in NOD mice (Tian *et al.* 1996a; Tisch *et al.* 1998). Secretion of cytokines from PBMC, without addition of antigen, revealed no differences between the groups at any of the sampling occasions. However, already one month after the first injection, *in vitro* stimulation with GAD<sub>65</sub> enhanced the levels of the Th2 associated cytokines IL-5 and IL-13 in the cell supernatants from the GAD-alum treated patients (Figure 19). None of the other studied cytokines differed between the two patient groups at the same time point.



**Figure 19.** *GAD<sub>65</sub>-induced IL-5, -13, -10, -17, IFN- $\gamma$  and TNF secretion (pg/mL) detected by multiplex fluorochrome technique (Luminex) in PBMC supernatants after 72 hours culture, in samples collected one, three and nine months after receiving GAD-alum (black circles; n=28) or placebo (white circles; n=30). GAD<sub>65</sub>-induced levels were obtained by subtraction of spontaneous secretion. Horizontal lines represent the median. Significant differences are indicated as p-values. Non significant differences are designated n.s.*

Interestingly, the highest levels of IL-5 and IL-13, at one month, were found in samples from children that responded best to the GAD-alum treatment, as defined by preservation of C-peptide. However, the limited number of patients regarded as clinical responders, made it difficult to draw conclusions from subgroup analyses due to the lack of statistical accuracy.

The recall response to GAD<sub>65</sub> was characterised by a broader range of cytokines in samples collected at three and nine months (Figure 19). In addition to the induced secretion of IL-5 and IL-13, enhanced levels of IL-17, TNF and IFN- $\gamma$  were detected at three months (Figure 19). Nine months after the first injection, the previously elevated cytokines, with the exception of TNF, remained higher in the GAD-alum group but was now also joined by IL-10 (Figure 19). These findings are consistent with our previous results including samples collected at the fifteen month visit (Ludvigsson *et al.* 2008).

An interesting observation was that the induced Th2-associated cytokine secretion increased over time, from one month to three and nine months after receiving GAD-alum treatment. None of the other cytokines showed the same pattern of early induction and continuous increase. Anti-inflammatory Th2 responses to a single  $\beta$ -cell antigen may result in spreading of Th2 immunity to unrelated autoantigens and protection from T1D, suggesting that Th2 responses may be a fundamental mechanism underlying antigen-based immunotherapies (Tian *et al.* 1997). It is important to highlight that the immune cell network is more complex than can be explained simply by a Th1/Th2 imbalance. For example, it has been shown that rapid and transient production of IFN- $\gamma$  by induced Tregs is essential to their *in vivo* function, by creating an environment that prevents further T cell activation (Wood and Sawitzki 2006). It has also been shown that protective vaccines induce CD4<sup>+</sup> cells that secrete several cytokines, whereas non-protective mostly trigger T cells that produce only one or two cytokines (Darrah *et al.* 2007; Aagaard *et al.* 2009; Lindenstrom *et al.* 2009). Our results could indicate that the immunomodulatory effect of antigen-based treatment is not only dependent on dominating Th2 and regulatory responses, but also on several other cytokines, which may be important for restoration of the immune balance.

The broad range of cytokine recall responses to GAD<sub>65</sub> was still present at the last two controls of the trial (21 and 30 months), in the GAD-alum-treated patients. Preliminary findings from the extended four year follow-up show a similar effect in addition to a better preservation of remaining insulin secretion in the treated group. This suggests an induction of

a GAD<sub>65</sub>-specific memory T cell population, able to rapidly produce cytokines and exert potent effector responses.

To investigate whether the effect of GAD-alum treatment was specific for GAD<sub>65</sub>, PBMC were stimulated with two other T1D associated antigenic peptides, IA-2 and insulin peptide. The responses were similar between the two groups before and after treatment, which supports the antigen-specific effect of GAD-alum.

### **Th2 and Treg associated markers increased over time**

Treatment with GAD<sub>65</sub> has been associated with the generation of Tregs in mice (Tisch *et al.* 1998). To explore whether an induced expression of markers associated with regulation could be observed in humans after GAD-alum therapy, we analysed the mRNA expression of the transcription factor FOXP3 and the cytokine TGF- $\beta$ , which are intimately associated with immune regulation. At all sampling occasions, from one month until the 30 month follow-up, an increased GAD<sub>65</sub>-induced expression of FOXP3 was observed in samples from the GAD-alum-treated patients compared with the placebo group. Equal expression of TGF- $\beta$  mRNA was detected between the two groups from baseline to the nine month visit. At 15 months, an induced expression was observed in the GAD-alum group, which was found also at the 30 month visit. Furthermore, GAD<sub>65</sub>-induced FOXP3 mRNA expression increased over time, similarly to the increased secretion of IL-5 and IL-13.

### **GAD-alum reduced cell activation**

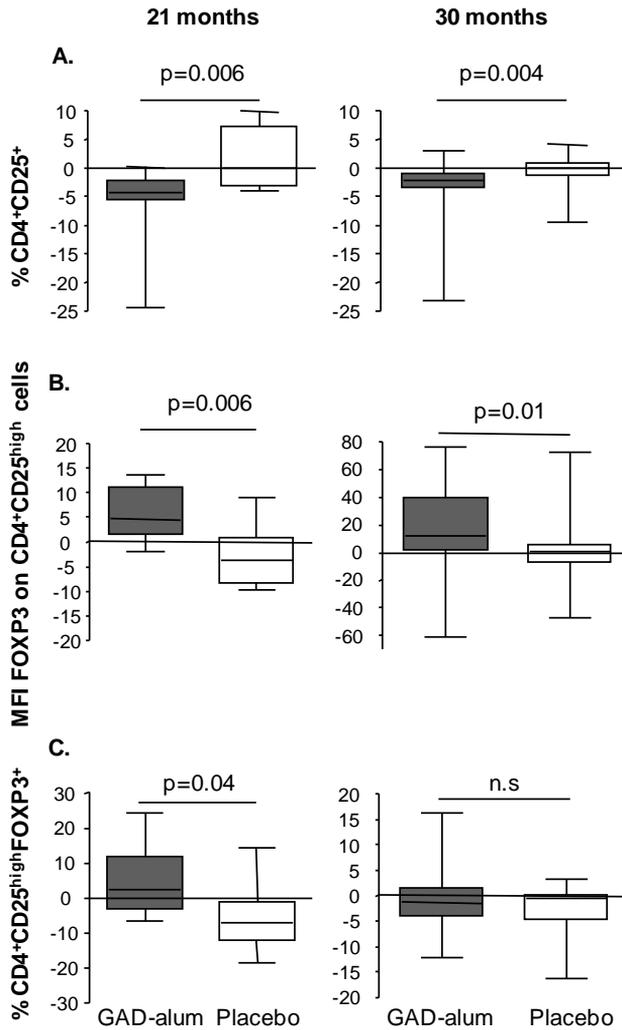
We have previously shown that GAD-alum treatment had an immunomodulatory effect in patients with recent-onset T1D, detected 15 months after the initial injection was given (Ludvigsson *et al.* 2008). To better understand how different subtypes of T cells were affected by GAD-alum treatment we analysed several different markers of interest by flow cytometry, with focus on CD4<sup>+</sup>CD25<sup>high</sup> cells. We observed reduced levels of CD4<sup>+</sup>CD25<sup>high</sup> cells co-expressing CTLA-4 and Neuropilin-1, markers that have been associated with both activation and regulation, in GAD-alum treated patients in comparison with placebo. To study the amount of receptors present on the cells, the median fluorescence intensity (MFI) is a beneficial tool. Within the CD4<sup>+</sup>CD25<sup>high</sup> gated cell population we found a lower MFI of CD25 in samples from GAD-alum-treated individuals, already three months after the initial injection. Since CD25 is up-regulated after T cell activation, we believe that this could indicate reduced activation of effector cells. Interestingly, the low expressions of the above

mentioned cell populations found in GAD-alum group were also detected in healthy children, supporting the hypothesis of a reduced inflammation and progression towards a normalised immunological phenotype after antigen therapy.

### **GAD-alum treatment induced GAD<sub>65</sub>-specific CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells**

As the study progressed, fewer differences were observed between the groups. In light of our previous findings, indicating a GAD<sub>65</sub>-specific effect of GAD-alum, PBMC collected at 21 and 30 months were stimulated *in vitro* with GAD<sub>65</sub> prior to flow cytometry analyses. No differences between the two patient groups were observed in cells cultured with medium alone. The stimulation with GAD<sub>65</sub> resulted in lower frequencies of CD4<sup>+</sup>CD25<sup>+</sup> cells in combination with an enhanced percentage and MFI of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells in GAD-alum-treated patients compared with the placebo group (Figure 20).

Although no functional studies were performed during the trial due to sample limitation, it is possible that the GAD<sub>65</sub>-induced population of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells in GAD-alum-treated patients identifies a T cell population with regulatory features, expanded in the periphery. Antigen-specific Tregs have been shown to be induced after anti-CD3 monoclonal antibody treatment in NOD mice (Belghith *et al.* 2003). In humans, the same treatment induced the expression of FOXP3 on CD8<sup>+</sup> T cells compared with untreated individuals (Bisikirska *et al.* 2005). Thus, our finding suggests a similar effect after GAD<sub>65</sub>-vaccination in human T1D.



**Figure 20. Flow cytometry analyses of  $GAD_{65}$ -induced expression of  $CD4^+CD25^+$  cells in samples collected 21 and 30 months after the initial injection. The  $GAD_{65}$ -induced expression of the different markers was calculated by subtraction of the expression in PBMC cultured in medium alone for each individual sample. (A) Percentage (%) of  $CD4^+CD25^+$  cells; (B) median fluorescence intensity (MFI) of FOXP3 on  $CD4^+CD25^{high}$  cells and (C) percentage of  $CD4^+CD25^{high}FOXP3^+$  cells at 21 (GAD-alum  $n=14$ ; placebo  $n=9$ ) and 30 months (GAD-alum  $n=17$ ; placebo  $n=17$ ). The box plot indicates the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles and the whiskers comprise the remaining data. Significant differences are indicated as  $p$ -values. Non significant differences are designated n.s.**

## **Tregs are associated with Th2 cytokine secretion**

It was interesting to find that the GAD<sub>65</sub>-induced expression of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells was strongly associated with secretion of IL-5, -10 and -13 in the GAD-alum group, but not in the samples from children who received placebo (Table III). Similarly, the induced expression of FOXP3 in GAD-alum treated patients observed by flow cytometry, was confirmed by mRNA analysis, and was also correlated with the secretion of IL-5, -10 and -13. In addition, TGF-β mRNA expression was also induced in GAD-alum-treated patients compared with placebo and correlated with IL-5 and IL-13.

An anti-inflammatory role of Th2 cytokines in the prevention of diabetes in NOD mice have been proposed (Tian *et al.* 1996a; Fox and Danska 1997). Administration of β-cell autoantigens, such as GAD<sub>65</sub> and insulin, induces Th2 and Tr1 like cells and prevents diabetes onset in NOD mice (Muir *et al.* 1995; Tian *et al.* 1996b; Tisch *et al.* 1998; Seifarth *et al.* 2003). Tregs can act as bystander suppressors by inducing anti-inflammatory cytokines, such as IL-4 and IL-10 (Chatenoud *et al.* 2001). All studied cytokines, i.e. IL-5, -6, -10, -13, -17, IFN-γ, and TNF, with the exception of IL-17, were inversely correlated with CD4<sup>+</sup>CD25<sup>+</sup> cells in samples from GAD-alum-treated patients (Table III). These findings, together with our observation of a lower percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells in the same group, suggests that the treatment reduced CD4<sup>+</sup> cell activation in parallel with a GAD<sub>65</sub>-specific induction of Th2 and regulatory associated cytokines. The results are in line with our findings of a GAD<sub>65</sub>-specific Th2 and regulatory immune response in the GAD-alum-treated group four weeks after the first injection of GAD-alum (Paper III). A broader range of cytokines was induced by GAD<sub>65</sub> at the later sampling occasions (Paper III; Ludvigsson *et al.* 2008), which is confirmed in this paper.

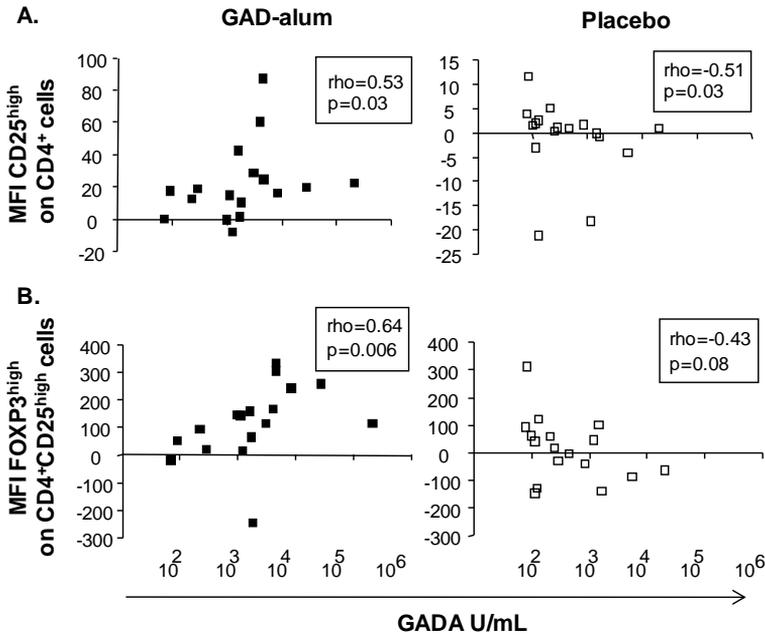
**Table III. Correlations between cytokines and CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells.** Correlation between GAD<sub>65</sub>-induced secretion (pg/mL) of interleukin (IL) -5, -6, -10, -13, -17, interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF) and GAD<sub>65</sub>-induced percentage (%) of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells in samples from GAD-alum and placebo patients collected 21 months after the initial injection. Significant differences are indicated as p-values and correlation coefficient as rho. Non significant differences are designated n.s.

Cytokine pg/mL		% CD4 <sup>+</sup> CD25 <sup>+</sup>		% CD4 <sup>+</sup> CD25 <sup>high</sup> FOXP3 <sup>+</sup>	
		GAD-alum	Placebo	GAD-alum	Placebo
<b>IL-5</b>	rho	-0.81	n.s	0.89	n.s
	p	<b>=0.001</b>		<b>=0.007</b>	
<b>IL-6</b>	rho	-0.64	n.s	n.s	n.s
	p	<b>=0.02</b>			
<b>IL-10</b>	rho	-0.73	n.s	0.79	n.s
	p	<b>=0.007</b>		<b>=0.04</b>	
<b>IL-13</b>	rho	-0.78	n.s	0.93	n.s
	p	<b>=0.003</b>		<b>=0.003</b>	
<b>IL-17</b>	rho	n.s	n.s	n.s	n.s
	p				
<b>IFN-<math>\gamma</math></b>	rho	-0,71	n.s	n.s	n.s
	p	<b>=0.01</b>			
<b>TNF</b>	rho	-0,77	n.s	0,85	0,82
	p	<b>=0.003</b>		<b>=0.02</b>	<b>=0.02</b>

### Tregs are associated with GADA levels

Patients with high GADA levels, at the time of inclusion in the study, responded better to the GAD-alum treatment (Chéramy *et al.* Manuscript under submission). Therefore, we wanted to investigate whether the GADA levels were associated with the expression of CD4<sup>+</sup>CD25<sup>high</sup> cells. Interestingly, at 30 months, GADA levels correlated with the GAD<sub>65</sub>-induced MFI of CD4<sup>+</sup>CD25<sup>high</sup> cells in GAD-alum treated patients (Figure 21 A). An inverse correlation was found in the placebo group. Moreover, the FOXP3<sup>high</sup> MFI in CD4<sup>+</sup>CD25<sup>high</sup> cells also

correlated with GADA in the treated group (Figure 21 B), similarly to the MFI of CTLA-4<sup>high</sup>. The significance of our observations is unclear, but B cell function is much more complex than producing antibodies (Gray *et al.* 2007). Among others, B cells act as antigen-presenting cells, secrete IL-10 and TGF- $\beta$  and might also be involved in Treg development and their recruitment to sites of inflammation (Mann *et al.* 2007).



**Figure 21. Correlation between GADA titres in serum and T cell markers by flow cytometry analyses in samples collected at 30 months.** GADA levels (U/mL) correlated with GAD<sub>65</sub>-induced median fluorescence intensity (MFI) of (A) CD25 on CD4<sup>+</sup>CD25<sup>high</sup> cells and (B) FOXP3<sup>high</sup> on CD4<sup>+</sup>CD25<sup>high</sup> cells in samples from GAD-alum (n=17) and inversely in placebo (n=17) patients. Significant differences are indicated as p-values and the correlation coefficient as rho.

Our results indicate that treatment with GAD-alum in T1D children reduces T cell activation and possibly pancreatic inflammation by expansion of GAD<sub>65</sub>-specific CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells. However, several questions regarding functional capability of these cells remain to be investigated.

## Summary & Conclusion

The immune system is composed of a complex network of cells that communicate with each other by different mechanisms and mediators. This communication needs to work properly in order for the immune system to maintain tolerance to self, but still be able to mount forceful responses to foreign antigens. Different regulatory mechanisms are of vital importance for maintaining a proper balance. If the immune system fails to suppress autoreactive cells, which are present in the majority of people, autoimmunity will occur. In the case of T1D, a misdirected attack against the pancreatic  $\beta$ -cells progressively diminishes the insulin production. Currently, there is no cure for T1D and the need for new therapies is obvious. Several different strategies have been investigated to find a way to prevent or delay the onset or progression of disease. In this thesis, I report immunological findings from treatment with Nicotinamide and GAD<sub>65</sub>. In addition, observations of the immunological profile before and after diagnosis of T1D are described.

We have found that individuals at increased risk of developing T1D had an elevated secretion of IFN- $\gamma$ . This suggests an aggressive Th1 cell-mediated immune response, probably directed against the pancreatic  $\beta$ -cells during the pre-diabetic phase. Close to the onset of clinical disease, the IFN- $\gamma$  secretion diminished. This could possibly be an effect of the reduced amount of remaining  $\beta$ -cells that release autoantigen and subsequently does not trigger the inflammatory process to the same extent.

Reduced secretion of chemokines and expression of Th1 associated chemokine receptors was found in newly diagnosed T1D children in comparison with healthy children. This proposes that the Th cell function is impaired in diabetics but could also indicate a selective recruitment of T cells to the pancreas. Cytotoxic T cells with memory phenotype were reduced at T1D onset, but with duration of disease this cell population increased to levels similar to that of the healthy individuals. In contrast, Tc cells with a naïve and migratory phenotype were elevated at diagnosis, possibly reflecting an active generation and recruitment of cells to the inflamed pancreas or perhaps a reduced function of the Tc cells. Speculatively, with duration of disease and as the metabolic control is improved after diagnosis and treatment with insulin, the immune system normalises and the peripheral memory T cell pool grows, as the recruitment of effector memory cells to the pancreas decrease.

Phenotyping the T cell subpopulations of the T1D patients was partly performed in order to better understand how duration of disease might affect responsiveness to immunotherapy and to GAD-alum treatment in particular. The induced levels of Tc cells in the periphery of recent-onset patients could be a clue in understanding why these patients are the ones that respond best to the treatment. I believe that development of tolerance by antigen-based immunotherapy is possibly induced more efficiently in patients with a high proportion of naïve cells in the peripheral blood, which still have not encountered their antigen. Injecting GAD<sub>65</sub> under the skin, where there are plenty of antigen presenting cells, together with the adjuvant alum, which favours Th2 skewed responses, promotes that the antigen is presented in a friendlier environment than in the inflamed pancreas. Thereby, the naïve Tc cells might be more prone to be primed towards protective Th2 and regulatory responses to the antigen, instead of destructive ones.

We have found that GAD-alum treatment in T1D patients resulted in an early GAD<sub>65</sub>-specific immune response with a predominant Th2 and regulatory profile. The recall response observed later during the trial was characterised by a broader range of cytokines, which is a phenomenon observed in other studies of vaccines. Interestingly, only the Th2 and regulatory responses increased over time in response to GAD<sub>65</sub>, indicating a specific immune deviation with long term immunological effect of the treatment. The GAD-alum treatment also expanded a cell population with regulatory phenotype specific for GAD<sub>65</sub>, in parallel with a reduction of activated Th cells. This could indicate that the treatment induced antigen-specific Tregs, which in turn suppressed the autoreactive response and inflammation in the pancreas. The previously reported clinical findings indicate a better preservation of endogenous insulin secretion in patients treated with GAD-alum, which supports our hypothesis of a reduced autoimmune destruction of pancreatic  $\beta$ -cells.

Even though Nicotinamide was ineffective in preventing development of T1D in individuals at increased risk, we were able to detect some immunological effects in suppression of the cell mediated Th1 like response, in the small group of Swedish patients included in our study. Possibly, another dose of Nicotinamide and/or in combination with another agent could give improved effects. At present, GAD-alum seems to be a promising new treatment for T1D. The ongoing phase III trials, including larger study populations, are based on the results from the Phase IIb study. Hopefully many of the research questions that have originated from our results will be answered in the future studies.

## **Future perspectives**

The effect of GAD-alum treatment on the chemokine and chemokine receptor expression still remains to be analysed. This will be done in an effort to increase the understanding of the immunological effects of antigen-based immunotherapy. New markers, with focus on memory and Treg cells, have been included in the four year follow-up of the Phase IIb study. Preliminary results show a persistent GAD<sub>65</sub>-specific effect in the treated patients and it will be interesting to see how the memory and Treg cell pool is affected in more detail. Further, the expression of CD4, CD25, Neuropilin-1 and CTLA-4, have been analysed in relation to effect of GAD-alum treatment. We will now investigate if the expressions of these markers are affected by duration of T1D. This study will hopefully further increase the knowledge of how duration of disease might have an impact on immune intervention.

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