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GAD_{65} AS AN IMMUNOMODULATOR IN TYPE 1 DIABETES

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ABSTRACT

Type 1 diabetes (T1D) is caused by a deficiency of insulin as a result of an autoimmune destruction of the pancreatic β-cells. A possibility to preserve remaining β-cells in children with newly diagnosed T1D is of great importance since sustained β-cell function is recognized to result in reduced end-organ complications. Glutamic acid decarboxylase 65 (GAD_{65}) is one of the major antigens targeted by self-reactive T cells in T1D, and immunomodulation with GAD_{65} formulated in aluminum (GAD-alum) has been considered both in prevention and treatment of T1D. Results from a Phase II trial have shown clinical effect of subcutaneous injections with GAD-alum, this was unfortunately not fully confirmed in the following larger Phase III trial which therefore was closed after 15 months. The general aim of this thesis was to study the immunomodulatory effect of GAD-alum-treatment in children with T1D participating in the Phase II and Phase III trials. We hypothesized that treatment with GAD-alum contributes to the preservation of residual insulin secretion through deviation of the GAD_{65}-specific immune response from a destructive to a protective process, accompanied by a shift from T helper (Th) 1 towards a predominant Th2 profile. In the Phase II trial, GAD-alum-treated patients responded with an early GAD_{65}-specific Th2 skewed cytokine secretion, with highest IL-5 and IL-13 secretion in clinical responders. Also, the CCR4/CCR5 ratio indicating balance between Th2/Tc2 and Th1/Tc1 responses, increased in treated patients. The recall response to GAD_{65} was characterized by a wide range of cytokines, but the relative contribution of each cytokine suggests a shift towards a more pronounced Th2-associated profile over time. Induction of a CD4+ cell subset upon GAD_{65}-stimulation 4 years after treatment, suggesting clonal expansion of the memory T-cell compartment upon antigen re-challenge, was seen in parallel to a persistent GAD_{65}-specific cytokine response. Finally, even if the phase III trial failed to reach the primary endpoint at 15 months, a subgroup analysis showed that the treatment had an effect on preservation of residual insulin secretion, but the effect was not seen until after 30 months. Taken together, these results suggest that GAD-alum treatment might exert its effect through induction of an early Th2 skewed immune response which tends to deviate away from a destructive Th1/Tc1 response upon GAD_{65} re-challenge, and generation of GAD_{65}-specific memory T cells that produce cytokines and exert effector responses which may be important for regulating GAD_{65} immunity. Continued research to better understand how immunomodulation with autoantigen modifies T-cell responses and also which patients are suitable for treatment, is crucial for optimizing future intervention trials using β-cell antigens.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Typ 1 diabetes (T1D) karakteriseras av kroniskt förhöjt blodsocker till följd av insulinbrist. Näst efter Finland har Sverige världens högsta förekomst av T1D hos barn under 15 år. Vad som orsakar sjukdomen är oklart, och av hittills oförklarliga skäl ökar antalet nyinsjuknade kontinuerligt. T1D tillhör gruppen autoimmuna sjukdomar, vilket innebär att de insulinbildande β-cellerna i bukspottkörteln attackeras av det egna immunsystemet. Framförallt anses T-hjälpar (Th)1-celler bidra till den inflammatoriska process som uppstår medan Th2-celler anses spela en beskyddande roll. Attacken är riktad mot insulin och andra proteiner i β-cellerna, såsom glutaminsyradekarboxylas (GAD_{65}) och tyrosinfosfatas. Vi har i en klinisk fas II studie visat att injektioner med GAD_{65} formulerat med adjuvantet alum (GAD-alum) hos barn med TID leder till bevarad β-cellsfunktion. Den kliniska effekten sågs tillsammans med en specifik effekt på immunsystemet, vilket kan vara förklaringen till den skyddande effekten på insulinproduktionen. Den lyckade fas II studien resulterade i en mer omfattande fas III studie, som inkluderade 334 barn med T1D från kliniker i hela Europa. Tyvärr uppnådde inte studien det förväntade kliniska resultatet 15 månader efter behandling, och avslutades därför i förtid.

Det övergripande syftet med mina fyra delarbeten var att studera hur behandling med GAD-alum påverkar immunsystemet, för att på så sätt öka kunskapen om immunologiska mekanismer och identifiera biomarkörer kopplade till klinisk effekt. Bevarad insulinproduktion är ytterst viktig för människor med T1D eftersom det hjälper dem att bättre kontrollera sin sjukdom och minskar risken för långtidskomplikationer. Vår hypotes är att behandling med GAD-alum bidrar till att bevara β-cellerna och därmed bibehålla den egna insulinproduktionen. Denna process skulle kunna ske via immunomodulering av det GAD_{65}-specifika cellsvaret; från att vara destruktivt till att verka beskyddande. Resultaten visar att de patienter som behandlats med GAD-alum uppvisar en tidig Th2-profil när cellerna stimuleras i provrör med GAD_{65}, dessutom har de patienter som svarar bäst på behandlingen högst nivåer av Th2-associerade budbärarmolekyler s.k. cytokiner. Vi visar även att kemokiner och dess receptorer, som är viktiga för hur cellerna rör sig, är påverkade och visar en övervikt mot Th2. Dessa fynd stödjer hypotesen att en beskyddande effekt induceras genom en övergång från det destruktiva Th1 till ett mer skyddande Th2 svar. Fyra år efter behandling finns fortfarande celler hos de behandlade patienterna som aktiveras av GAD_{65} och som uttrycker både Th1- och Th2- cytokiner och även andra proteiner, varav en del är viktiga för en stark bibehållen

minnes T-cellpopulation. Slutligen kan vi påvisa att även om fas III studien inte uppnådde det primära målet 15 månader efter studiestarten så finns det kliniska effekter som är märkbara, fast först efter 30 månader. Dessa effekter sågs parallellt med en cytokinprofil som över tid gick från att vara associerad med både Th1 och Th2 till en mer uttalad Th2-profil.

Slutsatsen från denna avhandling är att behandling med GAD-alum hos barn med T1D leder till GAD_{65}-specifik påverkan på immunsystemet. Behandlingens kliniska effekt skulle kunna vara kopplad till en tidig Th2-associerad immunprofil och bildande av GAD_{65}-specifika minnesceller som utsöndrar en mängd olika cytokiner och andra proteiner som är viktiga för immunreglering.

LIST OF ORIGINAL PAPERS

This thesis is based on the following four papers, which will be referred to in the text by their Roman numerals;

Paper I

Axelsson S, Hjorth M, Åkerman L, Ludvigsson J and Casas R

Early induction of GAD_{65}-reactive Th2 response in type 1 diabetic children treated with alum-formulated GAD_{65}
Diabetes/Metabolism Research and Reviews 2010; 26(7):559-568

Paper II

Axelsson S, Hjorth M, Ludvigsson J and Casas R

Decreased GAD_{65}-specific Th1/Tc1 phenotype in children with type 1 diabetes treated with GAD-alum
Accepted for publication in Diabetic Medicine 2012

Paper III

Axelsson S*, Chéramy M*, Hjorth M, Pihl M, Åkerman L, Martinuzzi E, Mallone R, Ludvigsson J and Casas R

Long-lasting immune responses 4 years after GAD-alum treatment in children with type 1 diabetes
PLoS ONE 2011; 6(12):e29008.

*These authors contributed equally to this work

Paper IV

Axelsson S, Chéramy M, Åkerman L, Pihl M, Ludvigsson J and Casas R

Preserved C-peptide 30 months after GAD-alum treatment of children and adolescents with recent-onset type 1 diabetes, and its relation to immune markers
Manuscript

ABBREVIATIONS

acDC ELISpot	Accelerated co-cultured dendritic cell Enzyme-linked immunospot
APC	Antigen presenting cell
AUC	Area under the curve
C-peptide	Connecting peptide
Ct	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
FCS	Fetal calf serum
FOXP3	Forkhead box P3
FSC	Forward scatter
GABA	γ-aminobutyric acid
GAD_{65}	Glutamic acid decarboxylase
GADA	Glutamic acid decarboxylase autoantibodies
GAD-alum	Aluminum-formulated GAD_{65}
HLA	Human leukocyte antigen
IA-2	Insulinoma-associated antigen-2
IA2A	Insulinoma-associated antigen-2 autoantibodies
IAA	Insulin autoantibodies
IFN	Interferon
IL	Interleukin
LADA	Latent autoimmune diabetes in adults
MHC	Major histocompatibility complex
MMTT	Mixed meal tolerance test
NOD	Non obese diabetic
PBMC	Peripheral blood mononuclear cell
PHA	Phytohemagglutinin
RT-PCR	Reverse transcription polymerase chain reaction
SSC	Side scatter
T1D	Type 1 diabetes
Tc cell	Cytotoxic T cell
T_{CM} cell	Central memory T cell
TCR	T cell receptor
T_{EM} cell	Effector memory T cell
TGF	Transforming growth factor
Th cell	T helper cell
TNF	Tumor necrosis factor
Treg cell	Regulatory T cell
TTX	Tetanus toxoid
ZnT8	Zinc transporter 8

INTRODUCTION

TYPE 1 DIABETES

Definition and Diagnosis

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia which results from defects in insulin secretion or action [1]. Type 1 diabetes (T1D), previously known as insulin-dependent or juvenile diabetes is the predominant form during childhood [2]. The clinical diagnosis is often prompted by symptoms such as increased thirst and urine volume and weight loss. These symptoms result from the underlying hyperglycemia that is in turn caused by insufficient insulin secretion. Diagnosis of T1D is made based on glucose measurement and the criteria for diagnosis are, according to the American Diabetes Association (ADA), fasting plasma glucose of ≥ 7.0 mmol/l, or symptoms of hyperglycemia and a casual plasma glucose value of ≥ 11.1 mmol/l, or 2-h plasma glucose ≥ 11.1 mmol/l during an oral glucose tolerance test [3]. The test should be performed as described in a report by the World Health Organization [1].

Incidence

The incidence of T1D in children is highly variable among different ethnic populations, and has been well characterized by registry reports from the DIAMOND project group worldwide [4], the EURODIAB study group within Europe [5] and the SWEDIABKIDS study group within Sweden [6].

The highest incidence is found in Caucasian populations and the lowest rates are found in Asia and South America [4]. Second to Finland, Sweden has the highest incidence worldwide, reported as 41.9/100 000 in 2010 in the 0-14.9 age group [6]. A rapid increase in the incidence of T1D has been reported from many countries during the last decades [2], and in most reports, the incidence is steepest among the youngest children. A recent study however shows that the accelerating increase may tend to level off in Sweden [7].

Pathogenesis and Etiology

T1D is an autoimmune disease caused by autoreactive immune cells which destroy pancreatic insulin-producing β-cells in the islet of Langerhans, eventually leading to complete insulin deficiency [8]. The remaining islets contain cells with enlarged nuclei, variable numbers of degranulated β-cells and a chronic inflammatory infiltrate referred to as insulitis. The infiltrate consists predominantly of T cells, of which CD8+ cells dominate, but may also contain CD4+ cells, B cells and macrophages [9]. The cellular response is accompanied by a humoral response that includes autoantibodies against a wide array of β-cell antigens. Studies have shown inverse correlation of age with greater loss of insulin reserve at diagnosis, suggesting that T1D follows a more aggressive course in younger children [10-11].

Since the early 1920s, insulin has been used to treat diabetes. However, insulin treatment has no effect on the autoimmune process and despite replacement therapy, long-term complications including retinopathy, nephropathy, neuropathy and cardiovascular disease causes substantial morbidity and mortality [12].

Genetic risk

Several genes have been shown to predispose for T1D. Of particular importance are genes located within the human leukocyte antigen (HLA) region [13], that accounts for approximately 45 % of genetic susceptibility for the disease [14]. There is a strong linkage of T1D to the highly polymorphic HLA class II immune recognition molecules DR and DQ located on chromosome 6. The protein products encoded by HLA class II genes are expressed on antigen presenting cells (APC) that capture and present processed peptide antigen to the T cell receptor (TCR), a central event in the initiation of any immune response. Nevertheless, only about 10 % of genetically susceptible individuals progress to clinical disease [15].

Polymorphisms in other genes, including insulin (INS), cytotoxic T lymphocyte antigen-4 (CTLA-4) and protein tyrosine phosphatase N22 (PTPN22) [16], are also believed to have effect on the risk of developing T1D. However, the fact that monozygotic twins are not uniformly concordant for disease development [17], implies that also environmental factors play a substantial role in the development of T1D.

Environmental factors

The rapid increase of T1D incidence indicates the significance of environmental and lifestyle changes. The hygiene hypothesis suggests that improved hygiene and living conditions have decreased the frequency of childhood infections, leading to insufficient stimuli to the immune system early in life [18]. This, in turn, could increase the risk of immune-mediated diseases, such as autoimmune and allergic disorders.

Certain viruses have been associated with β-cell destruction, including congenital rubella and enterovirus infections [19]. Viruses may cause β-cell destruction either by direct cytopathic effects on the target cells or indirectly by triggering or potentiating the autoimmune response [20]. Furthermore, an amino acid segment of GAD_{65} (aa 247-279) shares sequence similarity with the P2-C protein of Coxsackie B virus [21], which suggests the mechanisms of molecular mimicry to mediate the putative diabetogenic effect. Moreover, seasonal variations in diagnosis of T1D have been reported, with peaks during the autumn and winter months and decreases during the summer months [22], and viral infections have been suggested as a potential cause for this seasonality. A Diabetes Virus Detection Project (DiViD) trial is currently ongoing in Norway aiming to detect viruses and virus receptors within the insulin producing β-cells of the pancreas in patients with newly diagnosed T1D (NCT01129232).

It has been suggested that food content in early childhood may modify the risk of T1D later in life [23]. Short duration of breast-feeding and early exposure to complex dietary proteins has been implicated as risk factors for advanced β-cell autoimmunity or T1D. Administration of cow's milk early in life has been proposed to promote islet autoimmunity [24], and the TRIGR (Trial to Reduce IDDM in the Genetically at Risk, NCT00570102) trial test whether hydrolyzed infant formula compared with cow's milk-based formula decreases risk of developing T1D in children with genetic susceptibility [25]. Results from a 10 year follow-up of the TRIGR study recently showed that feeding with the hydrolysate formula was associated with a decreased risk of seroconversion to islet-cell antibodies [23].

β-cell stress has also been suggested as a risk factor for T1D development [26]. During periods of rapid growth e.g. puberty, the demand for insulin production increases, and this increased insulin production may result in β-cell stress and stimulation of the autoimmune process.

C-peptide and measurement of β-cell function

C-peptide is a connecting peptide in the middle of the proinsulin molecule that is co-secreted in equimolar concentration with insulin by the pancreatic β-cells, as a by-product resulting from the cleavage of proinsulin to insulin (Fig. 1). While the liver clears a significant portion of insulin, C-peptide does not undergo hepatic extraction [27]. Thus the plasma half-life of C-peptide is more than 30 min, compared to 3–4 min for insulin.

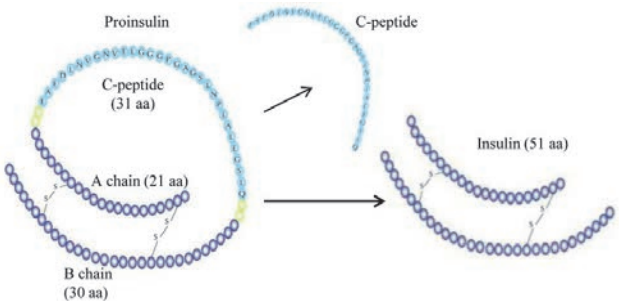

Figure 1. *Proinsulin is cleaved at two sites to form insulin and C-peptide. Insulin is composed of two peptide chains, the A chain and the B chain.*

Measurement of fasting and stimulated C-peptide in T1D patients is used in clinical settings as a measure of residual β-cell function [28]. During a mixed meal tolerance test (MMTT), blood samples for C-peptide are taken before and at 30, 60, 90 and 120 min after ingestion of a standardized liquid meal, to assess the patient's insulin production capacity. In normal subjects, the β-cells respond to oral glucose, fats and proteins with an increase in C-peptide levels, with a peak response usually within 90 minutes, and a return to baseline values after 120 minutes. In subjects with impaired β-cell function, the response is reduced, measured as area under the curve (AUC) and/or the peak value [27]. Even a modest residual insulin secretion, with stimulated C-peptide levels > 0.2 nmol/l, has been reported to provide clinically meaningful benefits in terms of reducing long-term complications [29].

Stages in the development of T1D

The fact that both genetic and environmental factors seem to have important roles in T1D development has led to the assumption that the autoimmune process leading to loss in insulin secretion is generally triggered by an environmental stimulus, but occurs primarily in those who are genetically predisposed for the disease. The linear β-cell decline hypothesis postulated by George Eisenbarth first in 1986 remains the most widely referenced benchmarked model for T1D (Fig. 2).

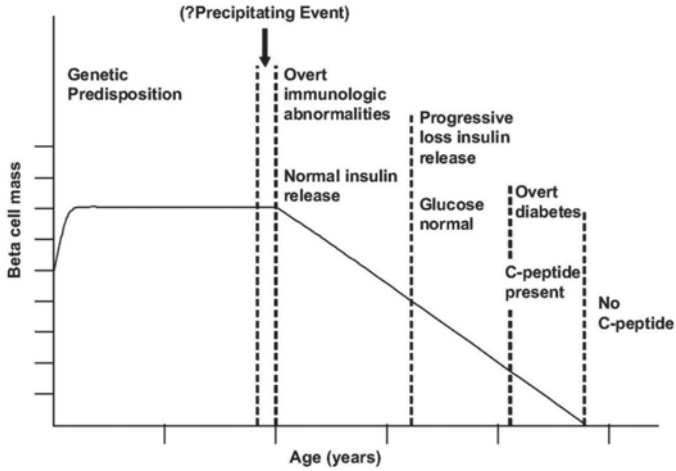

Figure 2. *Stages in the development of T1D. At some point, genetically susceptible individuals encounter certain environmental agents that initiate islet autoimmunity leading to a decay in β-cell mass, development of autoantibodies, hyperglycemia and eventually complete loss of C-peptide. Illustration adopted from [30].*

IMMUNOLOGY OF T1D

Autoantigens and Autoantibodies

The clinical manifestation of T1D is preceded by a preclinical phase during which islet autoantibodies appear as the first detectable sign of immune system activation against the islets. Thus, seroconversion is an early detectable sign of an ongoing autoimmune response [31]. The main autoantibodies in T1D are reactive to four islet autoantigens: glutamic acid decarboxylase (GAD_{65}), insulinoma-associated antigen-2 (IA-2), insulin and zinc transporter 8 (ZnT8).

The most extensively studied β-cell autoantigen is GAD_{65}, first discovered in 1982 [32]. GAD_{65} is an enzyme found in central and peripheral nerves as well as in pancreatic β-cells, that converts glutamic acid to γ-aminobutyric acid (GABA) [13]. Approximately 70-80 % of newly diagnosed T1D patients have autoantibodies to GAD_{65} (GADA). In 1996, the 979 amino acid insulinoma-associated protein 2 (IA-2), was identified [33] and similar to GAD_{65}, expressed in pancreatic islets and neurons. Approximately 55-80 % of newly onset T1D children have autoantibodies to IA-2 (IA2A). Autoantibodies to endogenous insulin (IAA), present before exogenous insulin treatment, were first described in 1983 [34]. This is usually the first autoantibody to appear in young children as a sign of β-cell destruction [35]. The most recently described T1D autoantigen is ZnT8, which is a member of the large cation efflux family that facilitates accumulation of zinc from the cytoplasm into intracellular vesicles. ZnT8 has been suggested as a component for providing zinc to storage processes in insulin-secreting pancreatic β-cells [36]. Autoantibodies to ZnT8 are present in 60–80 % of new-onset T1D, and both titers as well as the prevalence of ZnT8A increase with age [37].

In more than 95 % of patients with T1D, one or more types of islet autoantibodies may be detected at the clinical onset of disease [38]. In general, GADA positivity is rather stable, whereas IA2A tends to decrease with disease duration, and IAA cannot be usefully measured after initiation of insulin therapy. Approximately 1 % of healthy controls have detectable levels of autoantibodies to IA-2, GAD_{65}, or insulin. While a single autoantibody may represent non-progressive β-cell autoimmunity, the appearance of multiple antibodies is a marker of a progressive autoimmune destruction. The Diabetes Prevention Trial-Type 1 has reported that the risk of T1D in individuals with three or more autoantibodies was more than 50 % after 5 years [39].

T cells

T cells originate from hematopoietic stem cells in the bone marrow. They migrate and mature in the thymus where they undergo two selection processes: positive selection that permits survival of T cells whose TCR are capable of recognizing self-MHC (major histocompatibility complex) molecule, and negative selection that eliminates T cells that react too strongly with self-MHC plus self-peptide [40]. T cells that survive this selection process leave the thymus and circulate continually from the blood to peripheral lymphoid tissues. However, despite negative selection in the thymus, significant numbers of autoreactive T cells still escape to the periphery, capable of causing autoimmune diseases when immune regulation fail.

T cells fall into two major classes with different effector functions: T helper (Th) and cytotoxic T (Tc) cells, distinguished by surface expression of the glycoprotein CD4 or CD8, respectively. CD4+ cells bind to the MHC class II molecule expressed on APC, i.e. dendritic cells (DC), macrophages and B cells, while CD8+ cells recognize the MHC class I molecule expressed on all nucleated cells. Human MHC molecules are referred to as HLA.

A T cell with a receptor specific for a presented antigen will bind to the HLA/peptide complex and receive its first signal for activation. The T cell receives a second signal through binding of CD28 to CD80/86 (B7 molecules) expressed on the APC. Upon activation CTLA-4 is up-regulated and out-competes CD28 in binding affinity to CD80/86, generating an inhibitory signal and down-regulation of T-cell responses. Naïve CD8+ T cells require more co-stimulation to become activated compared to naïve CD4+ cells. This can be assisted by cytokines e.g. interleukin (IL)-2, released from CD4+ cells [40].

Antigen encounter induces T-cell proliferation, yielding approximately 1000 times more descendants with identical antigenic specificity that acquire effector functions and home to sites of inflammation [41]. Depending on local cytokine milieu, the Th cells develop into different phenotypes (Fig. 3) [42]. Most effector cells die after the antigen is cleared, but a few antigen-experienced memory cells remain for long-term protection.

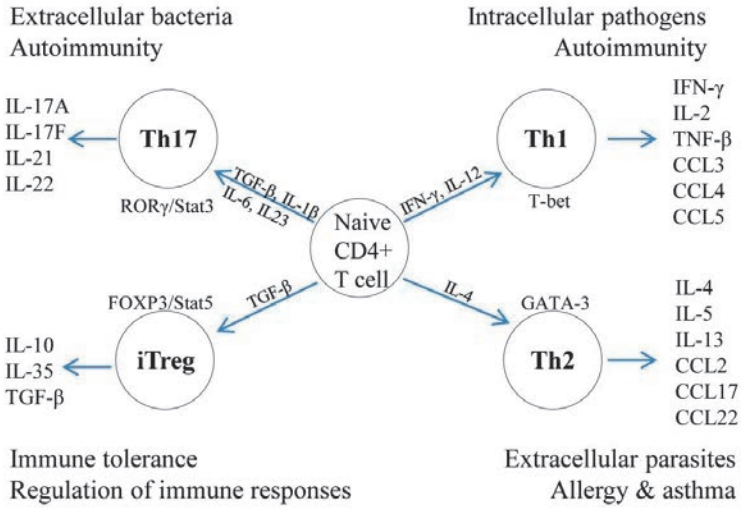

Figure 3. *Summary of the CD4+ T helper cell fates: their functions, their secretion products, their characteristic transcription factors and cytokines critical for their fate determination. Modified from [42].*

T helper cells

In 1986, Mosmann and colleagues defined two sub groups of Th cells with contrasting and cross-regulating cytokine profiles, namely Th1 and Th2 cells [43]. The functional significance of Th1- and Th2-cell subsets is their distinct patterns of cytokine secretion which lead to strikingly different T-cell actions. Mouse Th1 cells secrete interferon (IFN)-γ, IL-2 and tumor necrosis factor (TNF)-β, which are responsible for activating macrophages and Tc cells at the site of infection and are thereby mediators in cell-mediated immunity against intracellular pathogens [43]. In contrast, Th2 cells produce IL-4, IL-5 and IL-13 that activate eosinophils and induce B-cell antibody production to eliminate extracellular pathogens. Responses of Th1 and Th2 cells are mutually inhibitory. In humans however, the cytokine production is not as tightly restricted to a single subset as in mice, and the Th1/Th2 paradigm is clearly an over-simplification. Lately, this paradigm has been updated to include a more recently identified subset called Th17 cells, that mainly produce IL-17A IL-17F, IL-21, and IL-22 [44]. The cytokines IL-1β, IL-6, transforming growth factor (TGF)-β and IL-23 are essential for their development. Th17 cells have a pro-inflammatory effect and are believed to be important in the host's defense against infections and to be associated with the development of autoimmune diseases [45].

An imbalance between the Th1- and Th2-cell subsets has been suggested as a key determinant in establishing islet pathology in T1D [46]. T cells mediating β-cell destruction in recent-onset T1D patients are predominantly of Th1 cell phenotype secreting large amounts of IFN-γ [47]. Furthermore, T cells in patients with T1D exhibit polarization toward a Th1-type response to islet autoantigens *in vitro*, whereas non-diabetic control subjects display a Th2/Treg bias [48]. A recent study has demonstrated an increase of IL-17-secreting T cells in children with new-onset T1D, which suggests a role for this pro-inflammatory cytokine in the pathogenesis of disease [49].

Cytotoxic T cells

Similar to their Th-cell counterparts, distinct Tc-cell subsets have been established in mouse models [50] and in humans [51]. Analogous to the Th1/Th2 terminology, these subsets are termed Tc1 and Tc2, and have also been shown to produce Type-1 cytokines or Type-2 cytokines, respectively [52]. CD8+ T cells are among the first to infiltrate pancreatic islets in T1D. Histological studies of pancreas have documented significant islet Tc cell infiltration in recently diagnosed diabetic patients [53]. Also, β-cell epitope-specific CD8+ T cells are present in the peripheral blood in recent-onset diabetes patients [54], however they appear to shift in frequency and immunodominance during disease progression.

Regulatory T cells

In 1995, another subset of naturally occurring CD4+ was identified and characterized in mice; the CD4+CD25+ regulatory T cells (Treg) [55]. A few years later regulatory CD4+CD25^{high} T cells were also described in humans [56], representing 1-6 % of the total peripheral CD4+ T cell population [57]. A defining feature of Treg cells is their ability to inhibit proliferation of CD4+CD25- effector T cells [58], thus Treg cells play a crucial role for the suppression of autoreactive T cells. Such suppression is mediated by cell contact-dependent mechanisms, for example by inhibiting the induction of IL-2 mRNA in responder cells [59].

Further, Treg cells express forkhead box P3 (FOXP3), a master regulator belonging to a large family of transcription factors, necessary for their development and function [60]. Although FOXP3 expression has been shown to be up-regulated after *in vitro* activation of human non-regulatory CD4+ T cells [61-62], FOXP3 still remains as a commonly used marker for Tregs. In addition to the thymus derived Treg cells, conventional CD4+CD25- cells can turn on

FOXP3 and differentiate into a regulatory population, so called induced Tregs, when stimulated with antigen in the presence of a certain cytokine environment, e.g. TGF-β [63-64].

Both Treg frequency and function have been studied in patients with T1D. Whereas one initial study suggested that there might be a reduction in Treg frequency [65], following studies demonstrated that altered frequencies of Treg in peripheral blood are not associated with T1D [66-68]. In addition, there is no consensus regarding whether functional differences exist between T1D patients and healthy individuals in their Treg capacity to suppress proliferation of autologous effector cells [66, 69]. More recently cross-over co-culture experiments have demonstrated reduced susceptibility of effector T cells to regulation in human T1D, suggesting an increased resistance of effector T cells for Treg-mediated suppression as a mechanism for the defective regulation of autoimmunity in T1D patients [67, 70].

Memory T cells

Immunological memory results from clonal expansion and differentiation of antigen-specific lymphocytes, and at a second encounter with the antigen, memory T cells mount a fast and strong immune response. The leukocyte common antigen isoforms CD45RA and CD45RO have for long been used to identify human naïve and memory T cells, respectively [71]. Memory T cells contain two subsets, CD45RO+CCR7+ central memory (T_{CM}) and CD45RO+CCR7- effector memory (T_{EM}) cells, characterized by distinct homing capacity and effector function [72]. CCR7, expressed by naïve and T_{CM} cells, is an essential chemokine receptor for entry to lymph nodes [73]. T_{EM} cells have lost the constitutive expression of CCR7, instead they display chemokine receptors required for homing to inflamed tissues.

Upon re-stimulation, T_{EM} show low-activation threshold, vigorous proliferation and cytokine production with rapid kinetics. Proliferation of memory T cells can be driven not only by antigenic stimulation but also by cytokines. The cytokines IL-7 and IL-15, which are constitutively produced by a variety of cells, play an essential role for maintenance of both CD4+ and CD8+ T cells [72]. Also, IL-7 promotes the survival of naïve and memory T cells by up-regulation of the anti-apoptotic molecule Bcl-2 [74].

Autoantigen-specific memory CD4+ T cells are present early in progression to T1D, and a recent study has demonstrated the presence of memory CD4+ cells specific for $GAD65_{555-567}$ and $insulin_{A6-21}$ epitopes in both T1D patients and autoantibody positive children, but not in healthy subjects [75].

Cytokines

The microenvironment plays a crucial role for directing the T-cell response towards type 1 or type 2 cytokine secretion. Thus, detection of cytokines and chemokines is relevant to understand the extent and direction of immune responses. Cytokines are secreted by immune system cells to communicate and control local and systemic events of immune and inflammatory responses. Both the production of cytokines by cells and the action of cytokines on cells is complex: a single cell can produce several different cytokines, a given cytokine can be produced by several cell types and a given cytokine can act on one or more cell types [76]. Cytokine actions are usually local, but in some cases (notably macrophage-derived inflammatory cytokines e.g. IL-1, IL-6 and TNF-α) cytokines exert actions on distant organs.

A variety of cytokines is found in the insulitis lesions both in humans and in animal models of T1D where they play an important role in the destruction of β-cells. Both IFN-α and IFN-γ have been associated with β-cell destructive insulitis in humans [47, 77]. Further, IL-1, TNF-α, TNF-β and IFN-γ have been shown to impair insulin secretion and to be destructive to both rodent and human β-cells *in vitro* [78]. Since studies of the target organ are difficult in human T1D, most are performed in peripheral blood. Studies of cytokine secretion in peripheral blood mononuclear cells (PBMC) in T1D patients have shown increased Th1-associated cytokines (TNF-α, IFN-γ) in parallel with decreased or unchanged Th2-associated cytokines (IL-4, IL-10) compared with healthy subjects [79-80]. In addition, elevated serum levels of IL-1β and TNF-α have been detected in diabetic subjects at onset of clinical disease [81]. A study recently showed that monocytes from recent-onset T1D patients spontaneously secrete IL-1β and IL-6 which in turn induce potentially pathogenic IL-17/IFN-γ-secreting T cells, suggesting that the innate immune system in T1D may drive the adaptive immune system by expanding the Th17 population of effector T cells [82]. Paradoxically, administration of the Th1-associated cytokines IL-2 and IFN-γ and immunotherapies that induce Th1-type cytokine responses have been shown to prevent T1D, at least in murine models [83]. For instance, prevention of autoimmune diabetes by complete Freund's adjuvant in non-obese diabetic (NOD) mice is critically dependent on IFN-γ production and not on either IL-4 or IL-10 [84].

Chemokines and their receptors

Chemokines are small chemoattractant peptides with high structural homology. They have been classified into four groups; CXC, CX3C, CC and C, depending on the number and spacing of the first two conserved cysteine residues. Chemokines are produced by a wide variety of cell types in response to infection or agents that cause physical damage to a tissue [85]. Interplay between chemokines and their receptors is important for migration of lymphocytes between blood, lymph nodes and tissues [86], and during an immune response the lymphocyte recruitment and activation is dependent upon the local chemokine production and the cellular expression of the appropriate receptor.

Different effector CD4+ T cell subsets express different chemokine receptors; Th1 cells preferentially express CCR5 and CXCR3, whereas CCR3 and CCR4 are characteristic of the Th2 phenotype [41]. Further, secretion of CCR5 ligands e.g. CCL3, CCL4 and CCL5 has been associated with Th1 inflammatory responses [87] while ligands for CCR4, including CCL17 and CCL22 [88], may function as regulators of Th2 cells together with CCL2 [89]. Tc cells are less well characterized with regard to chemokine receptor expression, but the pattern and regulation of chemokine receptor expression of polarized subsets of Tc cells seem to overlap that of Th cells [90]. The promiscuity between chemokines and their receptors, with many chemokine receptors binding more than one chemokine with high affinity, suggests a complex network with effects depending on unique chemokine/chemokine receptor combinations [91].

Islet-specific Th1 cells have been shown to secrete multiple chemokines and promote rapid induction of autoimmune diabetes in mice [92]. Further, a study in patients with T1D has demonstrated elevated levels of CXCL10, a chemokine predominantly attracting T cells of the more aggressive Th1-type [93]. Furthermore, studies have revealed a decreased presence of PBMC expressing the chemokine receptors CCR5 and CXCR3 in newly diagnosed T1D patients compared with healthy controls [94-95], indicating a re-localization of chemokine receptor bearing PBMC into the inflamed pancreas.

IMMUNE INTERVENTION IN T1D

The term immune intervention refers to any therapeutic action that alters the immune system and if successful, cures a given immune-mediated disease [96]. In humans, the partial removal of autoantibodies through plasmapheresis in recent onset T1D patients in the early 1980s, preserved β-cell function to some extent [97]. Another early attempt was the use of the immunosuppressive drug Cyclosporine A [98]. This trial showed clinically positive results, however the lack of lasting effects together with severe side effects e.g. serious renal toxicity [99] limited the enthusiasm for use of broad-spectrum immune modulating agents. Since then, several clinical trials have taken place (*see below*), but sadly, no agent for reversing T1D has yet been identified.

At the same time, more than 195 different T1D intervention therapies have been successful in mice [100]. However, many preventive and therapeutic successes in the NOD mouse model translate inefficiently to humans. The lack of successful therapies in humans may be related to differences in the immune systems of mice and humans. Differences include key discrepancies in both innate and adaptive immunity, e.g. differential expression of Treg cell markers (e.g. FOXP3) and co-stimulatory receptors as well as variations in the balance of leukocyte subsets and Th1/Th2 differentiation [101]. Further, when comparing autoimmune diabetes between NOD mice and humans there are similarities such as a genetic predisposition, MHC-loci contribution and autoantigens, but also differences including incidence, gender bias and different humoral reactivity to β-cells [100]. These differences between mice and humans may have impact on the immune processes that drive the development of autoimmunity in the two species, and might also reflect the lack of therapeutic success in humans.

Monoclonal anti-CD3 antibody

The protein complex CD3 is located on the surface of T cells and is central to the initiation of T-cell activation. Preclinical studies have suggested several mechanisms by which non-FcR binding CD3-specific antibodies may produce a state of self-tolerance [102-103]. Modified non-FcR binding CD3 antibodies have also been tested in clinical trials. Two different antibodies; hOKT3γ1 (Teplizumab) and ChAglyCD3 (Otelixizumab) were used in clinical trials during the early 2000.

Teplizumab is a humanized anti-CD3 monoclonal antibody that has been mutated to greatly reduce Fc receptor and complement binding. A phase I/II randomized controlled study was initiated to test safety and efficacy of a single course of Teplizumab on the loss of insulin production in patients with new onset T1D. Results showed that treatment with Teplizumab prevented the loss of insulin production for 1 year after treatment at diagnosis, and the clinical effects persisted for at least 2 years [104-105]. Adverse events were common and often mild, but some patients also had serious adverse events. In 2009, a Phase III, randomized, double-blind, multinational, placebo-controlled study (Protégé, NCT00920582) was initiated to evaluate efficacy and safety of Teplizumab in children and adults with recent-onset T1D. The primary purpose of the study was to determine whether Teplizumab infusions lead to greater reductions in insulin requirements in conjunction with near normal blood sugar control compared to placebo. Unfortunately, primary outcome did not differ between groups after 1 year [106].

In a phase II trial with the humanized Fc-mutated anti-CD3 monoclonal antibody Otelixizumab, β-cell function was preserved in newly-onset T1D patients, and their insulin needs were decreased up to 4 years after treatment [107-108]. A phase III study (DEFEND-1, NCT00678886) was subsequently initiated in 2008 with a lower dose of Otelixizumab, but primary efficacy outcome of change in C-peptide after one year was not met [109].

Monoclonal anti-CD20 antibody (Rituximab)

Although T cells are most closely linked to T1D pathogenesis, B cells also seem to play a role [110] and B-cell depletion has been shown to reverse diabetes in mouse models [111-112]. Rituximab is a monoclonal antibody targeting the CD20 receptor unique to B cells, leading to depletion, and a recent study demonstrated that a four-dose course of Rituximab could preserve β-cell function over a 1-year period in newly diagnosed T1D patients [113].

DiaPep277

DiaPep277 is a stable peptide (aa 437-460) isolated from heat shock protein 60 (Hsp60). DiaPep277 acts through both toll like receptor (TLR) 2 and the TCR and promotes cell adhesion, inhibit migration and modulate cytokine secretion toward a Th2 anti-inflammatory cytokine profile, as opposed to Hsp60 that also acts in a pro-inflammatory manner via

activation of macrophages through TLR4 [114]. DiaPep277 treatment in adults with newly-diagnosed T1D has been shown to preserve residual C-peptide levels [115]. Unfortunately, phase II trials for immune suppression in children have been unsuccessful [116].

A phase III clinical trial is currently ongoing, including 457 newly diagnosed T1D patients aged 16-45 years (NCT00615264). Initial results from the trial are encouraging, with significant preservation of C-peptide levels in patients treated with DiaPep277 compared to the placebo arm [117]. The difference reflects a relative preservation of 23.4% compared to placebo. Additional analyses of clinical, efficacy and safety data from this study are ongoing. A second confirmatory, global Phase III study with DiaPep277 is currently being conducted (NCT01103284). Completion of patient recruitment for this study including 450 patients is anticipated by the first half of 2012.

Insulin

Initiation of clinical trials with insulin was based on a number of studies from NOD mice showing promising results using insulin, proinsulin and insulin$_{9\text{-}23}$ peptide [118-120]. Unfortunately, both intervention and prevention of T1D have failed to show clinical efficacy in humans [121-123]. However, oral insulin was retrospectively observed to delay T1D progression in the subgroup of participants with the highest IAA levels [123].

Anti-IL1 receptor antagonist (Anakinra)

The pro-inflammatory cytokine IL-1β has an important role in the pathogenesis of T1D and is, alone or in combination with other cytokines, cytotoxic to pancreatic β-cells [124]. In 2007, a clinical trial showed that blockade of the IL-1 pathway through the use of an IL-1 receptor antagonist (Anakinra), improved glycemia, β-cell secretory function and reduced markers of systemic inflammation in type 2 diabetes [125]. A randomized clinical trial of the effect of Anakinra on the insulin secretion in newly diagnosed T1D patients is currently ongoing (NCT00711503) [126], with the hypothesis that anti-IL-1 treatment as add-on therapy to conventional insulin therapy will preserve or enhance β-cell function. Estimated primary completion date is May 2012.

Since 2001, Anakinra is approved by the Food and Drug Administration (FDA) for the use in rheumatoid arthritis where it has an acceptable risk/benefit profile with more than 100,000 patients treated [126].

CTLA-4-immunoglobulin fusion protein (Abatacept)

CTLA-4, a homologue of CD28, is an essential negative regulator of T-cell immune responses. Abatacept (CTLA-4-immunoglobulin fusion protein) selectively binds to CD80 and CD86 on APC, thereby blocking the interaction with CD28, thus interfering with the early phases of T-cell activation, proliferation, and survival [127-128]. The effect of Abatacept has been evaluated in recent-onset T1D, and was shown to postpone reduction in β-cell function over 2 years, suggesting that T-cell activation still occurs at the time of clinical diagnosis. Despite continued administration of Abatacept over 2 years, the decrease in β-cell function with Abatacept was parallel to that with placebo after 6 months, which might indicate that T-cell activation wane with time [128].

GAD_{65} AS AN IMMUNOMODULATOR

GAD_{65} is a protein of 585 amino acids encoded at chromosome 10p11 [129], and is present in pancreatic β-cells as well as neurons (Fig. 4). GAD_{65} is an enzyme that converts glutamic acid to GABA, a major inhibitory transmittor substance stored in small neurotransmitter vesicles. The identification of GAD_{65} as an autoantigen of T1D began in 1982, when a 64 kD protein was detected in plasma from T1D patients [32]. Following studies showed that antibodies in sera from newly-diagnosed T1D patients were directed against this islet cell protein [130], and further biochemical characterizations led to the identification of the protein GAD_{65} [131]. As mentioned, GAD_{65} is one of the major antigens targeted by self-reactive T cells in T1D. Preclinical studies in NOD mice demonstrated that destruction of pancreatic β-cells was associated with T cells recognizing GAD_{65} [132].

Lessons from the NOD mouse

The majority of data on potential interventions with GAD_{65} has been derived from studies in mice. In 1994, one study demonstrated that intraperitoneal injection of GAD_{65} in NOD mice delayed the onset of diabetes compared to controls [133]. Later GAD_{65} was found to induce

antigen-specific Th2 responses and inhibit progression of β-cell autoimmunity [134], and that the induction of anti-inflammatory Th2 responses to a single β-cell antigen resulted in spreading of Th2 immunity to unrelated autoantigens and a reduced long-term disease incidence [135]. Another study showed that the inhibition of disease progression was mediated through induction of GAD_{65}-specific Treg cells [136].

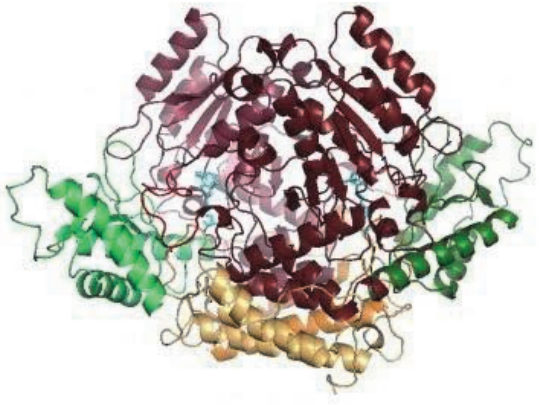

Figure 4. *Dimeric structure of GAD_{65}. Illustration modified from [137].*

The GAD_{65} vaccine

In 1994, the Swedish pharmaceutical company Diamyd Medical licensed the rights to GAD_{65} as the active substance in the antigen-based diabetes therapy Diamyd®. The company has performed a wide range of Good Laboratory Practice (GLP)-compliant animal safety studies to support the clinical use of recombinant human (rh) GAD_{65} formulated in aluminum adjuvant (GAD-alum) [138]. No adverse effects of $rhGAD_{65}$, other than local inflammation at the injection site, were observed in mice, rats, rabbits, marmosets or dogs when injected with or without adjuvant.

To assess the safety and tolerability after subcutaneous administration of separate and ascending doses, a clinical Phase I study was conducted in the UK by Diamyd Medical with 16 healthy male volunteers that received single subcutaneous injections of unformulated $rhGAD_{65}$, while 8 received placebo. No significant treatment-related adverse clinical effects were seen and no GAD_{65}, insulin, or IA-2 autoantibodies were induced. Consequently, it was concluded that the treatment was clinically safe and well tolerated, and a wide dose range of GAD-alum was subsequently proposed for clinical investigation.

Aluminum compounds have been widely used as human vaccine adjuvants for more than 70 years. It is known that the immunoadjuvant effect is associated with the induction of Th2 responses [139], but the mechanisms underlying this effect remain unknown. It likely involves various mechanisms including depot formation, increasing targeting of antigens to antigen presenting cells and non-specific activation of immune system [140].

Alum was selected as adjuvant for formulation with $rhGAD_{65}$ for clinical use for the following reasons: (i) aluminum salts are recognized to preferentially induce humoral rather than cellular immune responses, (ii) alum is used in several commercial vaccines (e.g. Diphteria, Tetanus and Pertussis (DTP)), (iii) historically alum has been the only adjuvant approved by the FDA [138]. Thus, the reason for using of alum in the formulation was to change the autoimmune response from a cellular towards a humoral response to GAD_{65}, in order to minimize the possibility of promoting cell-mediated β-cell destruction.

Clinical Phase II intervention trials

Following the extensive preclinical safety evaluation and the Phase I clinical trial with $rhGAD_{65}$, a randomized, double-blind, placebo-controlled, dose-finding Phase II study with GAD-alum was conducted in 47 latent autoimmune diabetes in adults (LADA) patients at the University Hospital MAS, Malmö and St Görans Hospital, Stockholm, Sweden [141]. The study was un-blinded after six months and the patients were followed for another four and a half years. The results supported the clinical safety of subcutaneous administration of GAD-alum, as well as its ability to increase both C-peptide levels (20 μg dose) and the CD4+CD25+ T cell subset in peripheral blood.

During 2005 to 2007 a randomized, double-blind, placebo-controlled Phase II study with GAD-alum was carried out, encompassing 70 children and adolescents aged 10-18 years with T1D recruited at 8 Swedish pediatric centers (NCT00435981). All participants had fasting serum C-peptide levels above 0.1 nmol/l and detectable GADA at inclusion. Patients were randomized to subcutaneous injections of 20 μg GAD-alum (n=35) or placebo (alum only; n=35) at day 0 and a booster injection 4 weeks later. Results showed that fasting and stimulated C-peptide secretion decreased significantly less over 30 months in GAD-alum treated patients compared to placebo [142]. No protective effect was seen in patients treated 6 months or more after receiving T1D diagnosis.

A four year follow-up study of 59 of the original 70 patients was later conducted to evaluate long-term efficacy and safety of GAD-alum intervention. Results showed that fasting C-peptide remained better preserved relative to placebo in patients with < 6 months T1D duration at baseline, and no treatment-related adverse events were reported [143].

In 2009, another phase II trial was initiated by the research consortium Type 1 Diabetes TrialNet, where patients received a third GAD-alum injection for a possible improved response to an additional booster dose (NCT00529399). Patients aged 3-45 years who had been diagnosed with T1D for less than 100 days were enrolled from 15 sites in the USA and Canada. The primary outcome was baseline-adjusted stimulated C-peptide secretion at 1 year. Unfortunately, results showed that treatment with two or three subcutaneous injections of GAD-alum, compared with placebo, did not affect the decline in insulin production during 1 year [144].

Clinical Phase III intervention trials

In 2008, a multi-centre, randomized, double-blinded European clinical Phase III trial was initiated (NCT00723411). The trial was performed in nine countries (Finland, France, Germany, Italy, Netherlands, Slovenia, Spain, Sweden and the UK) and aimed to investigate the impact of GAD-alum on T1D progression in newly diagnosed patients. Patients (n=334) aged 10-20 years with fasting C-peptide > 0.1 nmol/l and detectable serum GADA were enrolled within three months of T1D diagnosis. Patients received either four doses of 20 µg GAD-alum on day 1, 30, 90 and 270 (4D regimen), or two doses of GAD-alum on day 1 and 30 followed by two doses of placebo on day 90 and 270 (2D regimen), or four doses of placebo on day 1, 30, 90, and 270. The primary outcome was change in stimulated serum C-peptide between baseline and 15 months.

Results showed that the stimulated C-peptide level declined to a similar degree in all three treatment groups [145]. Further, GAD-alum treatment did not affect the insulin dose, glycated hemoglobin level or hypoglycemia rate, and adverse events were infrequent and mild in the three groups, with no significant differences. Thus, treatment with GAD-alum did not significantly reduce the loss of stimulated C-peptide or improve clinical outcomes over a 15-month period. The trial was therefore closed after 15 months, and the 30 months follow-up period was completed only for a minority of the patients. However, exploratory analysis showed a significant clinical effect of GAD-alum therapy for the 4D regimen, alone or

combined with 2D, in four subgroups; (i) males, (ii) patients with baseline Tanner puberty stage 2 or 3, (iii) patients with baseline insulin dose between 0.398 and 0.605 IU/Day/kg, and (iv) patients from non-Nordic countries.

In parallel to the European Phase III study, another Phase III study was conducted in the USA with the same purpose (NCT00751842). However, based on results from the recent Phase II and III trials with the same study drug, it was unlikely that this study would meet the intended efficacy endpoints. Therefore the primary focus of this study was changed to ensure that safety data is available for at least 6 months following the last injection of GAD-alum.

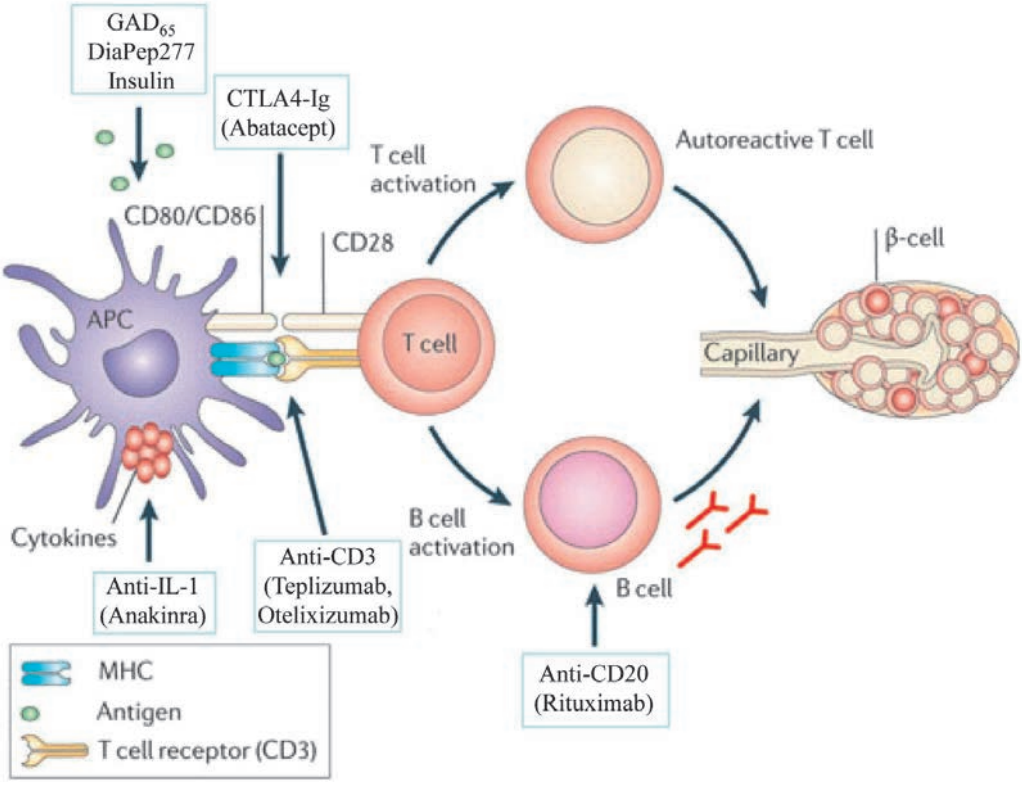

Figure 5. *Pathways and opportunities to intervene in T1D. Illustration modified from [146].*

Prevention trials

Multiple agents have been tested in patients at risk for developing T1D to examine the effect on preventing or reducing the incidence of disease. Studies aiming at prevention or delay of clinical T1D are critically dependent on the ability to identify individuals at risk for the disease. Several large scale multicenter clinical trials designed to prevent T1D have been conducted, and one of them is the European Nicotinamide Diabetes Intervention Trial (ENDIT) trial, where high-risk individuals were randomized to daily oral vitamin B3 or placebo for five years. However, the treatment did not prevent or delay development of T1D [147]. Another example is the Diabetes Prevention Trial-1 conducted in the USA and Canada that unfortunately also failed to demonstrate a benefit of oral or subcutaneous insulin therapy in preventing T1D [121, 123]. One more example of unsuccessful attempts is the Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP), that could not demonstrate a beneficial effect of daily intranasal insulin treatment in preventing or delaying T1D [148].

One ongoing trial that was initiated in 2009, is the Swedish double-blind randomized trial DiAPREV-IT (NCT01122446), aimed to determine the safety and effect of GAD-alum on the progression to T1D in children with multiple islet cell autoantibodies. This is the first prevention study with GAD-alum where the drug is given before onset of T1D. The primary objective is to demonstrate safety, and the secondary objective is to evaluate if the treatment may delay or halt the autoimmune process leading to manifest T1D in children with ongoing persistent β-cell autoimmunity. The trial is fully recruited and completion date is estimated to January 2017.

One prevention trial of infant nutrition is TRIGR, as already mentioned, conducted to test whether hydrolyzed infant formula compared with cow's milk-based formula decreases the risk of developing T1D in children with increased genetic susceptibility [25].

Immune correlates of clinical efficacy following therapeutic intervention

Characterization of antigen-specific T cells is a commonly used approach both for understanding the underlying pathogenic mechanisms of T1D and for assessing the efficacy of therapeutic intervention trials. However, in spite of numerous attempts using different therapies, a clear immunomodulatory effect on disease mechanisms remains unidentified and knowledge of immune correlates for clinical efficacy is limited. The autoantigen-specific

responses that represent biomarkers of disease e.g. GADA and GAD_{65}-specific T-cell responses, serve as potential biomarkers of clinical effect when GAD-alum is administered. Biomarkers could possibly also be useful to predict a patient's response to therapy.

During therapeutic intervention trials in humans in the last decade, studies have aimed to investigate the immune modulating properties. For instance, Diapep277 has been associated with increased cytokine production in response to therapy, dominated by IL-10, and production before treatment together with decreasing autoantigen-specific T-cell proliferation were associated with β-cell preservation [149]. Also, even if treatment with nasal insulin did not reduce the loss of residual β-cell function in adults with established T1D, treated patients displayed decreased *in vitro* T-cell IFN-γ responses to proinsulin, which suggests an induced immune tolerance to insulin [150]. Moreover, a beneficial effect of oral insulin treatment has been observed in individuals with high baseline IAA levels [123].

In the first Phase II GAD-alum trial in LADA patients, flow cytometry analysis revealed increased CD4+CD25+/CD4+CD25- cell ratio with a positive association to change in fasting C-peptide, suggesting an immunomodulatory mechanism for the treatment [141]. Serum cytokine levels were however not affected. Previous results from our group including patients from the Phase II GAD-alum trial with T1D children, showed that *in vitro* stimulation with GAD_{65} induced CD4+CD25^{high}FOXP3+ cells in the treated group, which may have the potential to reduce inflammation [151]. Further, a positive association between GAD_{65}-induced expression of CD4+CD25^{high}FOXP3+ cells and secretion of Th2 and regulatory cytokines was observed. Also, high baseline GADA levels were associated to more pronounced C-peptide preservation [152]. In order to improve β-cell antigen treatment, alone or in combination with other therapies, it is of utmost importance to learn more about the immunological effects.

HYPOTHESIS AND AIMS OF THE THESIS

The general aim of this thesis was to study the immunomodulatory effect of GAD-alum-treatment in children with T1D. We hypothesized that treatment with GAD-alum might contribute to preservation of residual insulin secretion through deviation of the GAD_{65}-specific immune response from a destructive to a protective process, accompanied by a shift from Th1 towards a predominant Th2 profile.

The specific aims were:

I. To clarify the immunomodulatory effect of GAD-alum shortly after treatment, with focus on cytokine secretion.

II. To further study the immunomodulatory effect of GAD-alum treatment focusing on chemokines and chemokine receptors.

III. To evaluate the long-term antigen-specific memory T- and B-cell responses in T1D children treated with GAD-alum.

IV. To characterize the immunomodulatory effect of GAD-alum, with and without additional injections, in recent onset T1D patients included in the Phase III trial, and to assess whether treatment preserved β-cell function in patients that completed the 30 months visit.

MATERIAL AND METHODS

STUDY POPULATIONS

The baseline characteristics for patients participating in the Phase II and III GAD-alum trials are given in Table I, Table II and Table III.

Table I. Baseline characteristics according to study group in the Phase II trial (Papers I & II)

Characteristic	GAD-alum n=35	Placebo n=34
Age (years)	13.8±2.3	12.8±1.9
Months since diagnosis	9.9±5.3	8.8±5.5
Gender distribution, n (%)		
Female	23 (66)	18 (53)
Male	12 (34)	16 (47)
HLA risk classification, n (%)		
High	18 (51)	16 (47)
moderate	9 (26)	7 (21)
Low	8 (23)	11 (32)
Tanner puberty stage, n (%)		
1	4 (11)	7 (21)
2+3	8 (23)	10 (29)
4+5	23 (66)	17 (50)
C-peptide (nmol/l)		
Fasting C-peptide	0.33±0.19	0.35±0.23
Stimulated C-peptide AUC	0.62±0.28	0.71±0.43
Glycated hemoglobin (%)	6.3±1.3	6.2±1.0
Insulin dose (IU/Day/kg)	0.66±0.30	0.66±0.28
Fasting plasma glucose (mmol/l)	9.4±4.0	8.8±3.3
Median GADA (Units/ml)	601	861
Median IA-2A (Units/ml)	125	552

Values are mean±SD unless stated otherwise. HLA, human leukocyte antigen; AUC, area under the curve; GAD-alum, alum formulated glutamic acid decarboxylase; GADA, Glutamic acid decarboxylase autoantibodies; IA2A, insulinoma-associated antigen-2 autoantibodies.
The Tanner puberty stage ranges from 1 to 5, with higher stages indicating more developed genitalia. HLA risk classification was based on HLA-DQ-A1* and -B1* alleles.

In Papers I and II, patient samples from the Phase II GAD-alum trial were included. The design of the trial is described elsewhere [142]. Briefly, 70 children and adolescents aged 10-18 years with T1D were recruited between February and April 2005 at 8 Swedish pediatric centers (Linköping, Stockholm, Göteborg, Halmstad, Malmö, Örebro, Jönköping and Borås).

All participants had a fasting serum C-peptide level > 0.1 nmol/l, detectable GADA levels and less than 18 months disease duration at inclusion. Patients were randomized to subcutaneous injections of 20 µg GAD-alum (n=35) or placebo (alum only; n=35) at day 0 and a booster injection 4 weeks later. One placebo patient was withdrawn from the study after one week, owing to confirmed infectious mononucleosis

To evaluate long-term efficacy and safety of GAD-alum intervention, patients and their parents were in 2009 asked whether they were willing to participate in a 4 year follow-up study. Of the original 70 patients included in the Phase II trial, 59 agreed to participate, of whom 29 had been treated with GAD-alum and 30 had received placebo [143]. Paper III includes samples from the 4 year follow-up.

Table II. Baseline characteristics according to study group, for patients that participated in the 4 year follow-up of the Phase II trial (Paper III)

Characteristic	**GAD-alum n=29**	**Placebo n=30**
Age (years)	13.6±2.4	12.8±1.9
Months since diagnosis	9.4±5.4	8.5±5.4
Gender distribution, n (%)		
Female	19 (65.5)	15 (50)
Male	10 (34.5)	15 (50)
C-peptide (nmol/l)		
Fasting C-peptide	0.3±0.2	0.4±0.2
Stimulated C-peptide AUC	0.6±0.3	0.7±0.4
Glycated hemoglobin (%)	6.2±1.3	6.2±0.9
Insulin dose (IU/Day/kg)	0.7±0.3	0.6±0.3
Fasting plasma glucose (mmol/l)	9.5±4.1	8.7±3.4
Median GADA (Units/ml)	539	786
Median IA-2A (Units/ml)	125	552

Values are mean±SD unless stated otherwise. AUC, area under the curve; GAD-alum, alum formulated glutamic acid decarboxylase; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies.

Table III. Baseline characteristics according to study group in the Phase III trial (Paper IV). Characteristics are given for the entire Swedish cohort and for the subgroup of patients who completed the 30 month visit.

	Swedish Subgroup			Entire Swedish cohort		
Characteristic	**4D**	**2D**	**Placebo**	**4D**	**2D**	**Placebo**
	n=14	**n=15**	**n=16**	**n=49**	**n=49**	**n=50**
Age (years)	13.2±2.4	13.3±2.4	13.4±2.7	13.3±2.1	13.3±2.2	13.4±2.5
Days since diagnosis	76.4±26.1	67.7±22.8	67.6±23.5	74.6±20.3	73.3±20.0	71.0±20.6
Gender distribution, n (%)						
Female	9 (64)	6 (40)	8 (50)	25 (51)	26 (53)	20 (40)
Male	5 (36)	9 (60)	8 (50)	24 (49)	23 (47)	30 (60)
HLA risk classification, n (%)						
very high	3 (21.3)	7 (47)	6 (38)	14 (29)	18 (37)	16 (32)
High	6 (43)	6 (40)	5 (31)	21 (44)	23 (47)	23 (46)
Moderate	3 (21.3)	2 (13)	4 (25)	8 (17)	4 (8)	8 (16)
Low	2 (14.3)	0 (0)	1 (6)	5 (10)	4 (8)	3 (6)
Tanner puberty stage, n (%)						
1	2 (14.3)	4 (27)	0 (0)	6 (12)	10 (20)	5 (10)
2+3	2 (14.3)	2 (13)	8 (50)	17 (35)	12 (25)	16 (32)
4+5	10 (71.3)	9 (60)	8 (50)	26 (53)	27 (55)	29 (58)
C-peptide (nmol/l)						
Fasting C-peptide	0.37±0.19	0.23±0.09	0.26±0.10	0.30±0.17	0.25±0.11	0.26±0.14
Stimulated C-peptide AUC	0.71±0.33	0.61±0.23	0.71±0.21	0.71±0.35	0.66±0.26	0.64±0.24
Glycated hemoglobin (%)	7.07±0.72	7.15±0.91	7.04±1.11	6.84±0.65	6.81±0.91	6.87±1.04
Insulin dose (IU/Day/kg)	0.64±0.26	0.71±0.27	0.51±0.23	0.59±0.29	0.62±0.31	0.55±0.23
Fasting plasma glucose (mmol/l)	6.81±1.92	6.32±2.15	5.87±1.24	6.28±2.16	5.95±1.74	5.69±1.19
Median GADA (Units/ml)	193	440	219	204	312	190
Median IA-2A (Units/ml)	408	667	943	472	620	736

Values are mean±SD unless stated otherwise. HLA, human leukocyte antigen; AUC, area under the curve; 4D, four dose regimen, 2D, two dose regimen; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies.
Data regarding HLA classification were missing for one patient in the 4D group.
The Tanner puberty stage ranges from 1 to 5, with higher stages indicating more developed genitalia. HLA risk classification was based on HLA-DQ-A1* and -B1* alleles.

Paper IV includes samples from 148 children and adolescents aged 10-20 years with T1D participating in the European Phase III trial described elsewhere [145], that were recruited between August 2008 and November 2009 at 20 Swedish pediatric centers. All patients had fasting C-peptide > 0.1 nmol/l, detectable GADA levels and less than three months disease duration at inclusion.

Patients were randomized to one of the following treatments: (i) four doses of 20 µg GAD-alum on days 1, 30, 90 and 270 (4D regimen), (ii) two doses of GAD-alum on days 1 and 30 followed by two doses of placebo on day 90 and 270 (2D regimen), (iii) four doses of placebo on days 1, 30, 90, and 270. Of the 148 subjects who were eligible and underwent randomization in Sweden (4D n=49, 2D n=49, placebo n=50), 45 patients completed the 30 months visit before the trial was closed (4D n=14, 2D n=15, placebo n=16). Baseline characteristics of the Swedish patients, as well as the subgroup that completed the 30 month visit, are representative of the whole European cohort.

PBMC ISOLATION AND IN VITRO STIMULATION

Two 9 ml Sodium Heparin Vacuette® tubes and one 4 ml serum gel Vacuette® tube were drawn from each patient at each study visit for immunological assays (Fig. 6). To avoid time-of-day differences, sample collection was performed during the morning hours. All samples were transported to Linköping within 24 h.

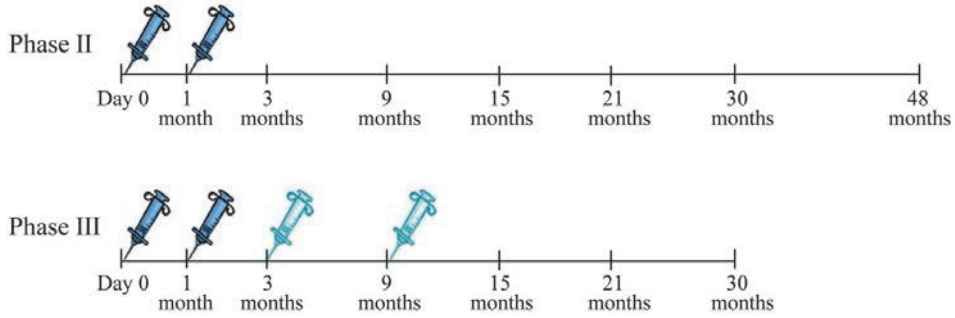

Figure 6. *Overview of sample collection in Phase II and III trials. In the Phase II trial, injections of GAD-alum or placebo were given at day 0 and 1 month. In Phase III, two additional injections were given; one at 3 months and one at 9 months, i.e. 4 dose regimen received GAD-alum whereas 2 dose and placebo received placebo injections. Blood samples were collected at day 0 and after 1, 3, 9, 15, 21 and 30 months, and also at 48 months in the Phase II trial.*

PBMC isolation

PBMC were isolated from sodium-heparinized venous blood samples using Ficoll Paque (Pharmacia Biotech) density gradient centrifugation (Paper I-III) or Leucosep® (Greiner bio-one; Paper IV) and were thereafter washed three times in RPMI 1640 medium (Invitrogen) supplemented with 2 % fetal calf serum (FCS; Gibco). Cells were either used directly for *in*

vitro stimulation (Paper III-IV) or cryopreserved in liquid nitrogen (Paper I-II) until used for *in vitro* stimulation. Serum was collected and stored at -70°C.

Cryopreservation and thawing process

Cryopreserved PBMC are commonly used when assessing immune responses in clinical trials, both for practical reasons and to minimize inter-assay variation as samples are often collected and studied longitudinally. To assess the suitability of cryopreserved PBMC for our assays, we performed a pilot study evaluating the effect of cryopreservation on spontaneous, antigen- and mitogen-induced cytokine and chemokine secretion using Luminex, and on mRNA expression of TGF-β and FOXP3 using real-time reverse transcription (RT)-PCR in PBMC from T1D children [153]. Our results indicated that cryopreserved PBMC from these patients remained suitable for assessment of both spontaneous, GAD_{65}- and mitogen-induced responses, even though their expression could differ from freshly handled cells. Thus freshly handled and cryopreserved samples should not be used in the same set of experiments.

In our assay, isolated PBMC were gently diluted in cold (+4°C) freezing medium consisting of 10 % dimethyl sulfoxide (DMSO; Sigma), 50 % FCS and 40 % RPMI 1640 medium, and distributed in aliquots at a cell density of 5×10^6 PBMC/ml in cryovials (Sarstedt). The vials were placed in -70°C in a pre-cooled (+4°C) Cryo 1°C Freezing Container containing isopropanol (Nalge Nunc International), to achieve a freezing rate of -1°C/min. The following day, vials were transferred and stored for approximately 12 months in liquid nitrogen. Cryopreserved PBMC were thawed in a +37°C water bath under continuous agitation and washed once in RPMI 1640 supplemented with 10 % FCS. Cell viability was determined by trypan blue exclusion prior to *in vitro* antigen stimulation.

In vitro stimulation

One million PBMC diluted in 1 ml AIM V medium supplemented with 20 μM β-mercaptoethanol (Sigma) were cultured at 37°C in 5 % CO_2 in medium alone or in the presence of stimulus (Table IV). After 72 hours (Papers I, II, III) or 7 days (Paper IV), the cell supernatant was collected and PBMC were re-suspended in RLT lysis buffer (Qiagen Sciences). PBMC lysates and cell supernatant aliquots were frozen at −70°C until used for real-time RT-PCR/PCR array and Luminex analyses, respectively.

Table IV. Final concentration of antigens used for *in vitro* stimulation

Stimuli	Concentration	Supplier	Paper
GAD_{65}	5 µg/ml	Diamyd Medical	I, II, II, IV
GAD-alum (vaccine formulation)	5 µg/ml	Diamyd Medical	I
Alum (placebo formulation)		Diamyd Medical	I
IA-2 $_{853-872}$ peptide	10 µg/ml	ProImmune	I,III,IV
Insulin $_{9-23}$ peptide	0.5 µg/ml	Sigma	I
TTX	100 ng/ml	Calbiochem	III, IV
PHA	5 µg/ml	Sigma	I,II

GAD_{65}, glutamic acid decarboxylase; GAD-alum, GAD_{65} formulated in aluminum; IA-2, insulinoma associated antigen 2; TTX, tetanus toxoid; PHA, phytohemagglutinin.

CYTOKINE AND CHEMOKINE ANALYSES

Detection of antigen-specific cells *ex vivo* is a great challenge due to low frequencies; only one in 30,000 or less CD4+ T cells in peripheral blood from patients with recent-onset T1D is GAD_{65}-specific [154], and activation and *in vitro* amplification of the GAD_{65}-specific T cells is crucial for detection. Cytokine and chemokine secretion was used to detect immune responses to GAD_{65} in the search for immune markers which might explain or predict clinical response to GAD-alum treatment.

Luminex

The microsphere-based flow cytometric assay developed by Luminex Corporation involves covalent coupling of a capture antibody on polystyrene microspheres, internally dyed with a red and an orange fluorophore. By varying the ratio of the two fluorophores, up to 100 different bead sets can be distinguished. Each bead set can be coupled to a different biological probe, in our setting the antibodies were directed against different cytokines and chemokines. Samples, standard and blank are incubated with the color-coded beads in a 96-well plate, thereafter a biotinylated detection antibody is added that binds to the analyte captured to the beads, and in the last step streptavidin-phycoerythrin (PE) is added to each well (Fig. 7). The bound microspheres are then passed though a flow chamber where a reporter laser (532 nm) excites the fluorescent molecules bound to the microsphere surface and a classification laser (635 nm) excites the fluorochrome held within the microsphere. Quantification of each analyte is based on the intensity of the fluorescent reporter signal.

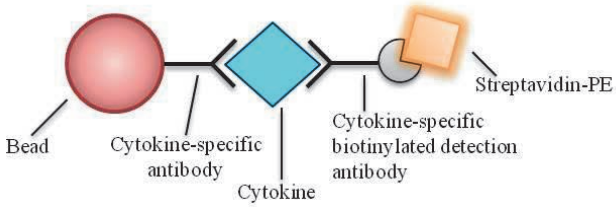

Figure 7. *Principle of the Luminex methodology. An antibody coupled bead binds to the analyte, a biotinylated detection antibody is added and thereafter streptavidin-PE.*

In our assay, acquisition conditions were set with a minimum of 100 beads per bead set. Raw data (median fluorescence intensity) were analyzed using the StarStation Software (Paper I-III) or the MasterPlex QT (Paper IV). A five-parameter curve fit was applied to each standard curve in order to obtain sample concentration values. A Bio-Plex™ (Papers I-III) or Bio-Plex Pro™ (Paper IV) cytokine panel (Bio-Rad Laboratories) was used according to manufacturer's instructions. The cytokines and chemokines analyzed in each paper are shown in Table V.

Luminex enables evaluation of multiple analytes simultaneously, compared to conventional methods e.g. Enzyme-linked immunosorbent assay (ELISA), which is a huge advantage in situations where sample volume is limited. The assay is sensitive and accurate since each fluorescent signal is the mean of 100 measurements of single microspheres, and each microsphere represents an assay by itself.

Table V. Analyzed cytokines and chemokines in each paper

Cytokine/Chemokine	Paper
IL-1β	III, IV
IL-2	III, IV
IL-5	I, III, IV
IL-6	I
IL-7	III
IL-10	I, III, IV
IL-12(p70)	I
IL-13	I, III, IV
IL-15	III
IL-17	I, III, IV
TNF-α	I, III, IV
IFN-γ	I, III, IV
TGF-β	IV
CCL2	II
CCL3	II
CCL4	II

Accelerated co-cultured dendritic cell (acDC)-amplified ELISpot assay

Enzyme-linked immunospot (ELISpot) serves as a sensitive tool to detect rare antigen-reactive T cells *ex vivo* by their ability to secrete cytokines. The assay allows visualization at a single cell level, making it possible to analyze antigen-specific cells of different phenotypes. Accordingly, the ELISpot assay provides both qualitative (type of secreted protein) and quantitative (number of responding cells) information.

In Paper III, detection of antigen-specific T-cell responses was performed with an accelerated co-cultured dendritic cell (acDC)-amplified ELISpot assay, as described elsewhere [155]. In the assay, antigen and DC-activating agents rapidly induce, pulse and mature DCs, thus lining up the sequential steps of T-cell activation within 48 h and amplifying antigen-specific responses. The utility of these acDC-based assays for immune monitoring of vaccination trials has been previously demonstrated [150].

Briefly, PBMC were washed twice in AIM-V medium and re-suspended in AIM-V medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Cells were seeded at 10^6/100µl/well in flat-bottom 96-well plates and stimulated with GAD_{65}, tetanus toxoid (TTX; Statens Serum Institut) or no antigen at 37°C in 5 % CO_2. After 24 h, 100 µl AIM-V medium containing TNF-α, IL-1β, prostaglandin E2 and IL-7 was added to each well and cells were cultured for another 24 h. Following this 48 h stimulation, non-adherent cells were washed, re-suspended in fresh AIM-V medium, seeded in quadruplicates at 1×10^5 cells/well and incubated for 6 h in 96-well PVDF plates (Millipore) precoated with anti-IFN-γ or anti-IL-4 antibodies (U-CyTech). Secretion of IFN-γ and IL-4 was visualized with a biotin-conjugated anti-IFN-γ or -IL-4 antibodies (U-CyTech), alkaline phosphatase-conjugated ExtrAvidin and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (both from Sigma). Spots were counted using a Bioreader 5000 Pro-SF (Bio-Sys). Means of quadruplicate wells were calculated and the results expressed as spot-forming cells (SFC)/10^6 PBMC after background subtraction. The cut-off for a positive response was set at 3 SD above the average basal reactivity.

GENE EXPRESSION ASSAYS

RNA isolation

In Paper I and III, total RNA was isolated from lysed PBMC according to the RNeasy 96 vacuum/spin protocol (Qiagen). The RNA was quantified by optical density (OD) measurements at 260 nm. Purity of RNA was ensured with an OD 260/280 ratio above 1.8. In Paper III, RNA integrity was confirmed using Agilent 2100 bioanalyzer (Agilent Technologies).

Real-time RT-PCR

Real-time RT-PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. In our assay, detection was possible due to a gene-specific probe which carries a reporter fluorochrome at the 5´ and a quencher at the 3´ ends. The method utilizes a polymerase to cleave the probe during PCR which separates the quencher from the reporter that allows the reporter to emit its energy and thereby generating a detectable signal. The amount of reporter signal increase is proportional to the amount of product for a given sample, and the higher the starting copy number of the target, the sooner a marked increase in fluorescence is observed. A threshold line that is set in the exponential phase of the amplification, and the cycle at which the sample reaches this level is called the Threshold cycle, Ct. Further, normalizing to a stably expressed reference (housekeeping) gene, that is representative of the complementary DNA (cDNA) concentration in a sample is a commonly used normalization approach.

In Paper I, gene expression of the transcription factor FOXP3 and the cytokine TGF-β was analyzed in PBMC, cultured for 72 h in AIM-V medium with or without GAD_{65}. In our assay, cDNA was synthesized with 7 ng/µl of total RNA using high-capacity cDNA archive kit (Applied Biosystems). FAM-labeled primers/probes were used to determine transcription levels of FOXP3 (Hs00203958) or TGF-β (Hs00171257) (Applied Biosystems), and VIC-labeled primers/probes were used for the housekeeping control 18s rRNA (4310893E) (Applied Biosystems). Together with TaqMan Universal PCR Master Mix (Applied Biosystems), the reaction mixture was amplified using the 7500 Fast Real-Time PCR System (Applied Biosystems). In addition, a no template control and an internal control were run in

each assay. The mRNA expression for each sample was calculated by subtracting the Ct value for the endogenous control from the Ct value for the target mRNA, expressed as ΔCt. Further, to calculate the GAD_{65}-induced mRNA expression, the ΔCt value for spontaneous expression (non-stimulated sample) was subtracted from the stimulated sample ΔCt value, to obtain the ΔΔCt value. The GAD_{65}-induced expression was then defined as relative transcription, based on the calculation $2^{-\Delta\Delta Ct}$.

PCR array

The PCR array constitutes of a 96-well plate that can contain up to 84 different primers of target genes, housekeeping genes and controls, and thereby enable simultaneous detection of expression of target genes. The system uses the SYBR Green dye to detect PCR products by binding double-stranded DNA formed during PCR. During the PCR, polymerase amplifies the target sequence which creates the amplicon. As the PCR progresses, more amplicons are created and since SYBR Green binds to all double-stranded DNA, the result is an increase in fluorescent intensity that is proportional to the amount of generated PCR product.

In Paper III, expression of 15 selected target genes was analyzed using a customized Human Gene RT2 profiler™ PCR array (SABiosciences), in PBMC cultured for 24 h in AIM-V medium with or without GAD_{65}. In our assay, each RNA sample was transcribed into cDNA with the RT2 First Strand Kit (SABiosciences). Templates were then combined with RT2 SYBR® Green/ROX™ qPCR Master Mix and loaded into each well containing the pre-dispensed gene-specific primer sets. ABI Prism 7900HT was employed for sequence detection, and sequence detection systems (SDS) version 2.3 (Applied Biosystems) was used to calculate Ct values. GAPDH and HPRT1 were used as housekeeping genes. An evaluation of the quality controls provided the relative levels of genomic DNA contamination and inhibition of either the reverse transcription or the PCR itself.

Relative gene expression was calculated with the ΔΔCt method, using the normalized ΔCt value of each sample, calculated by subtracting the average Ct value of the two housekeeping genes from the Ct value of the gene of interest. The spontaneous Ct value was thereafter subtracted from the Ct value of the GAD_{65}-stimulated sample. To calculate the ΔΔCt, the average ΔCt value of each gene in the placebo group was subtracted from the average Ct value of the corresponding gene in the GAD-alum group. The fold-change for each gene was calculated as $2^{-\Delta\Delta Ct}$.

FLOW CYTOMETRY

Flow cytometry allows measurement of multiple characteristics of single cells using light scatter properties and fluorescence properties of fluorescent probes with specificity to cellular constituents.

In Paper II, PBMC were stained immediately after isolation or cultured over night in AIM-V medium with or without GAD_{65} prior staining. 1×10^5 PBMC were stained with allophycocyanin (APC)-conjugated anti-CD4, peridinin chlorophyll (PerCP)-conjugated anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CCR5 and PE-conjugated anti-CCR4 (BD Biosciences). Isotype controls (BD Biosciences) were included to estimate the amount of non-specific binding. Lymphocytes were gated according to forward (FSC) and side scatter (SSC). Four-colour flow cytometry was performed with a BD FACSCalibur, and data was analyzed using Kaluza version 1.1 (Beckman Coulter).

In paper III, PBMC were cultured for 7 days in AIM-V medium with or without GAD_{65}. After incubation, 1×10^6 PBMC were stained with Alexa-700-conjugated anti-CD3, APC-Cy7-conjugated anti-CD4, PE-Cy7-conjugated anti-CD8, PE-Cy5-conjugated anti-CD45RA (BD Biosciences) and PE-conjugated anti-CCR7 (R&D Systems). Isotype controls (BD Biosciences) were included to estimate the amount of non-specific binding. Lymphocytes were gated by FSC and SSC, and the CD3+ events were plotted against side scatter to identify T cells. Flow cytometry was performed with a BD FACSAria, and data analyzed using Kaluza version 1.1.

PBMC PROLIFERATION ASSAY

Measurement of proliferative responses of human lymphocytes is a fundamental technique for the assessment of their biological responses to various stimuli, and proliferation determined by incorporation of ^3H thymidine into DNA, provides an estimate of cell proliferation indirectly by measuring DNA synthesis.

In paper III and IV cell proliferative responses were determined by ^3H thymidine incorporation assay. In our assay, PBMC were re-suspended at 1×10^6 cells/ml in AIM-V medium and incubated in triplicates (2×10^5 cells/well) in round-bottom 96-well plates with GAD_{65}, IA-$2_{853\text{-}872}$, TTX, phytohemagglutinin (PHA) or without antigen. After 3 days, cells were pulsed for 18 h with 0.2 μCi of ^3H thymidine/well (PerkinElmer), and thereafter

harvested. Proliferation was recorded using a Wallac MicroBeta counter and expressed as a stimulation index (SI), calculated as the mean of triplicates in presence of stimulus divided by the mean of triplicates with medium alone.

C-PEPTIDE MEASUREMENT

In the phase II GAD-alum trial, a two-hour MMTT was performed in accordance with a European study on estimation of β-cell function [28]. Serum C-peptide concentrations were measured at 0, 30, 60, 90, and 120 min after a mixed liquid meal (Sustacal®) ingestion (6 ml/kg body weight; maximum, 360 ml) using a time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit, Wallac). Results were validated by the inclusion of a C-peptide control module containing a high, a medium and a low-level control in each assay (Immulite, Diagnostic Products Corp.).

In the phase III GAD-alum trial, serum C-peptide analyses were performed by BARC Laboratories (Ghent, Belgium) with an Immulite 2000 C-peptide kit using an Immulite 2000 analyzer with calibration standards based on the World Health Organization's National Institute for Biological Standards and controls (Reference Standard 84/510).

In Papers I and II, the clinical effect of treatment was determined by changes in stimulated C-peptide AUC from baseline to 15 months (main study period). The GAD-alum treated patients were stratified into three groups; Responders (loss of AUC < 10 %), Intermediate responders (loss of AUC between 10 % and 65 %) and Non-responders (loss of AUC > 65 %).

In Paper III, clinical effect of treatment was defined by changes in stimulated C-peptide AUC from baseline to 48 months. GAD-alum-treated patients were divided in two subgroups; patients with a loss of C-peptide AUC ≤ 60 %, and patients with a loss of AUC > 60 %.

STATISTICAL ANALYSES

As the immunological markers were not normally distributed, non-parametric tests corrected for ties were used. Unpaired analyses were performed using the Mann-Whitney U-test, and correlations were analyzed with Spearman's rank correlation coefficient test. Differences within groups were analyzed by Friedman's test followed by Wilcoxon signed rank test. A probability level of < 0.05 was considered statistically significant. Calculations were

performed using SPSS version 17.0 for Windows (SPSS Inc) in Paper I, and PASW statistics version 18 for Windows (SPSS Inc) in Papers II, III and IV.

ETHICS

Collection of blood samples was performed after written informed consent was obtained from all patients and for those < 18 years old also their parents, in accordance with the Declaration of Helsinki. The studies were approved by the Research Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden.

All laboratory analyses were performed in a blinded manner.

RESULTS AND DISCUSSION

Cytokine and chemokine responses after GAD-alum treatment

Early induction of GAD_{65}-specific Th2 response in T1D children treated with GAD-alum

Between 2005 and 2007 we performed a Phase II clinical trial with GAD-alum in 70 children with T1D. In our first publication from this study in 2008, we reported clinical benefits of the treatment together with GAD_{65}-specific effects on the immune system including secretion of a wide range of cytokines, detected after *in vitro* stimulation of freshly handled PBMC samples collected 15 months after intervention [142]. At this point we did unfortunately not have the possibility to analyze cytokine secretion in freshly handled samples from the preceding study visits. To investigate whether the immunomodulatory effect of GAD-alum was apparent already close after treatment, preceding the changes reported at 15 months, we instead took advantage of the cryopreserved PBMC samples collected at early visits. Based on previous results in NOD mice [134, 136], we hypothesized that a Th2 predominant immune response would follow after the first injection of GAD-alum.

Our results showed that one month after the first injection, though prior to the booster injection, *in vitro* stimulation with GAD_{65} enhanced the levels of the Th2 associated cytokines IL-5 and IL-13 in GAD-alum-treated patients compared to placebo (Fig. 8), whereas the other analyzed cytokines did not differ between the two groups. Two months after the GAD-alum booster dose, not only the Th2-type cytokines were enhanced by *in vitro* stimulation with GAD_{65}, but also cytokines associated with Th1 (IFN-γ, TNF-α) and Th17 (IL-17) type immune responses. A transient Th2 polarization has also been observed after treatment with anti-CD3 monoclonal antibodies in NOD mice [156]. Although the recall response was associated with a wide range of cytokines, secretion of IL-5 and IL-13 continuously increased over time in the treated patients, and there was a correlation between GAD_{65}-induced IL-5 and IL-13 (Fig. 9). The generation and establishment of a predominant Th2 response to GAD_{65} might counteract proinflammatory factors, and generate an environment where autoreactive Th1 effector cells could be suppressed, thereby restoring immunological balance.

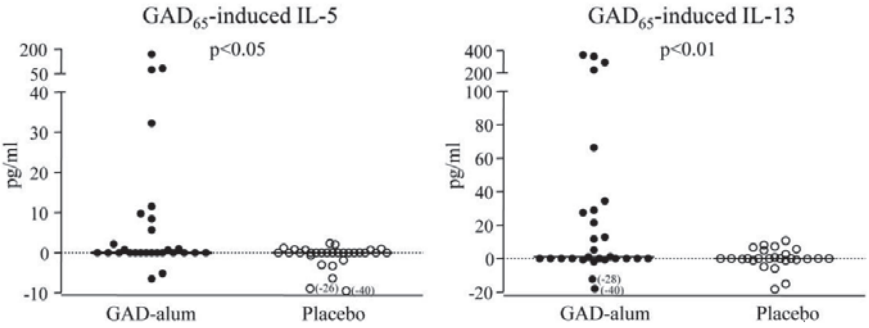

***Figure 8**. GAD_{65}-induced IL-5 and IL-13 secretion one month after receiving GAD-alum (black circles) or placebo (white circles). Cytokine secretion is given after subtraction of spontaneous secretion and expressed as pg/ml. Horizontal lines represent medians, outliers are indicated in brackets.*

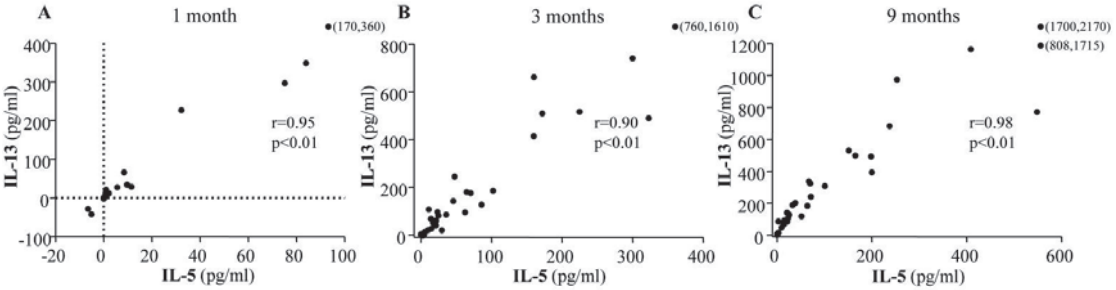

***Figure 9.** Correlation between GAD_{65}-induced IL-5 and IL-13 in GAD-alum treated patients at one (A), three (B) and nine (C) months after the first injection. Outliers are indicated in brackets. Significant differences are indicated as p-values and the correlation coefficient as r.*

We aimed to confirm the finding with an early GAD_{65}-specific Th2 skewed response in the following larger Phase III trial, including samples from the 148 Swedish T1D children using freshly handled PBMC samples. Unfortunately an early Th2 deviation could not be detected in the Phase III study, as both Th1 and Th2 associated cytokines increased in the treated patients at one month (Fig. 10). It is however important to note that neither the clinical effect that was observed in the Phase II trial was confirmed. Also, variations in cytokine detection between the studies could be explained by the shorter *in vitro* antigen-stimulation time as well as the usage of cryopreserved cells in the Phase II study.

In vitro recall responses to GAD_{65} were characterized by a wide range of cytokines

In line with our findings at the 15 month visit from the Phase II trial [142], the response to GAD_{65} included a wide range of cytokines at three and nine months. This result was confirmed in the Phase III trial where secretion of Th1, Th2 and Treg associated cytokines was detected already after one month and throughout the trial in response to GAD_{65}, both in patients receiving 2 doses of GAD-alum (2D) and in those receiving 4 doses (4D) (Fig. 10).

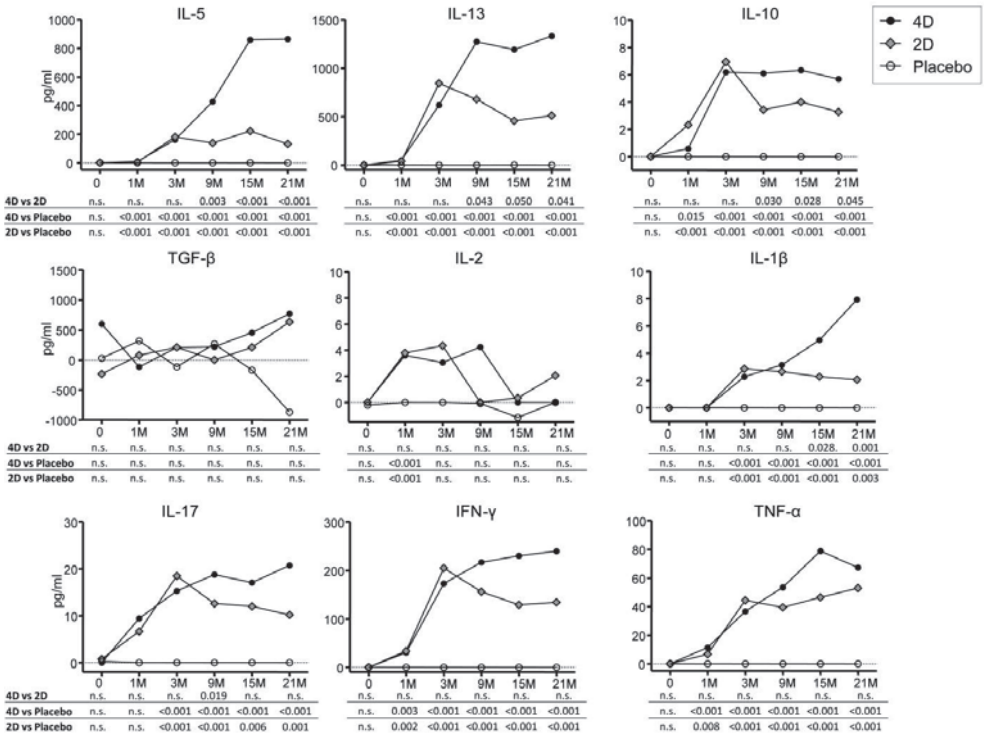

Figure 10. *Median secretion of IL-1β, IL-2, IL-5, IL-10, IL-13, IL-17, IFN-γ, TNF-α and TGF-β (pg/ml) from baseline to 21 months in the entire Swedish cohort, in patients receiving 4 doses of GAD-alum (4D, black circles), 2 doses of GAD-alum (2D, gray rhombuses) or placebo (white circles). GAD_{65}-induced cytokine secretion is given after subtraction of spontaneous secretion. Significant differences are indicated as p-values.*

Patients receiving 4D in the Phase III trial displayed higher secretion of several of the analyzed cytokines at 9, 15, 21 and 30 months compared to 2D regimen, which might be expected since two extra doses of GAD-alum were given, one at the 3 month visit and one at 9 months.

In contrast to our findings which demonstrated early Th2 associated cytokines in the Phase II trial, the cytokine profile tended to switch from a wide cytokine profile (including both Th1 and Th2 associated cytokines) towards a more pronounced Th2-associated profile over time in the phase III study, both in 2D and 4D regimen (Fig. 11). However, when assessing the relative contribution of each cytokine in the Phase II trial, the profile was similar to the one seen in the Phase III trial (unpublished data). Thus, even if the early cytokine response differs between the two trials, the overall cytokine profile resembled.

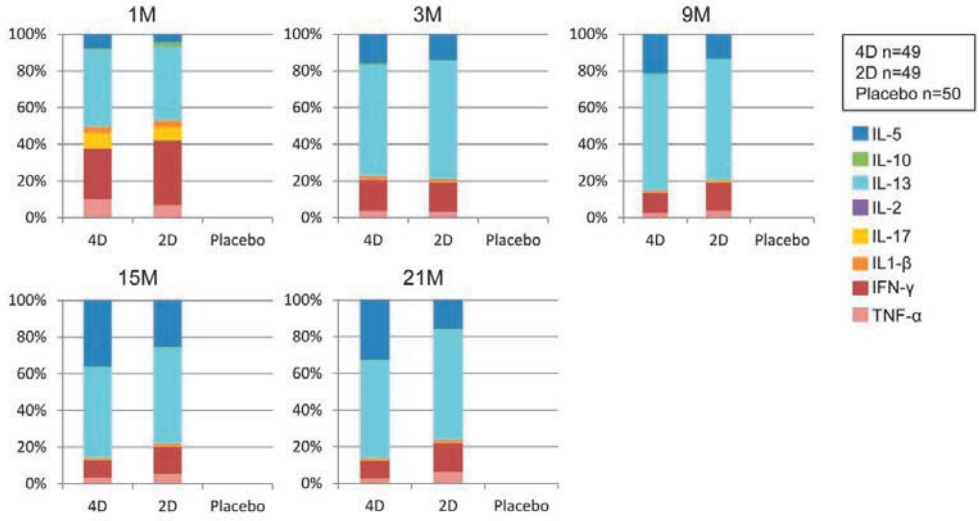

Figure 11. *Bar chart illustrating the relative contribution (%) of each cytokine to the GAD_{65}-induced secretion detected by Luminex from 1 month to 21 months follow-up in patients receiving 4 doses of GAD-alum (4D), 2 doses of GAD-alum (2D) or placebo.*

Four years after GAD-alum treatment in the Phase II trial, a wide range of cytokines was still detectable after *in vitro* stimulation with GAD_{65} (Fig. 12). In addition, our acDC ELISpot assay revealed that the number of cells secreting IL-4, a cytokine difficult to detect with the Luminex assay, was significantly increased in the GAD-alum group compared to placebo.

Finally, even though GADA positivity, which indicates an ongoing autoimmunity towards GAD_{65}, was an inclusion criterion in the GAD-alum trials, *in vitro* responses to GAD_{65}-stimulation were in general low in all baseline samples and also in the placebo groups throughout both trials. This highlights the great challenge to detect antigen-specific cells *ex vivo* due to their low frequencies.

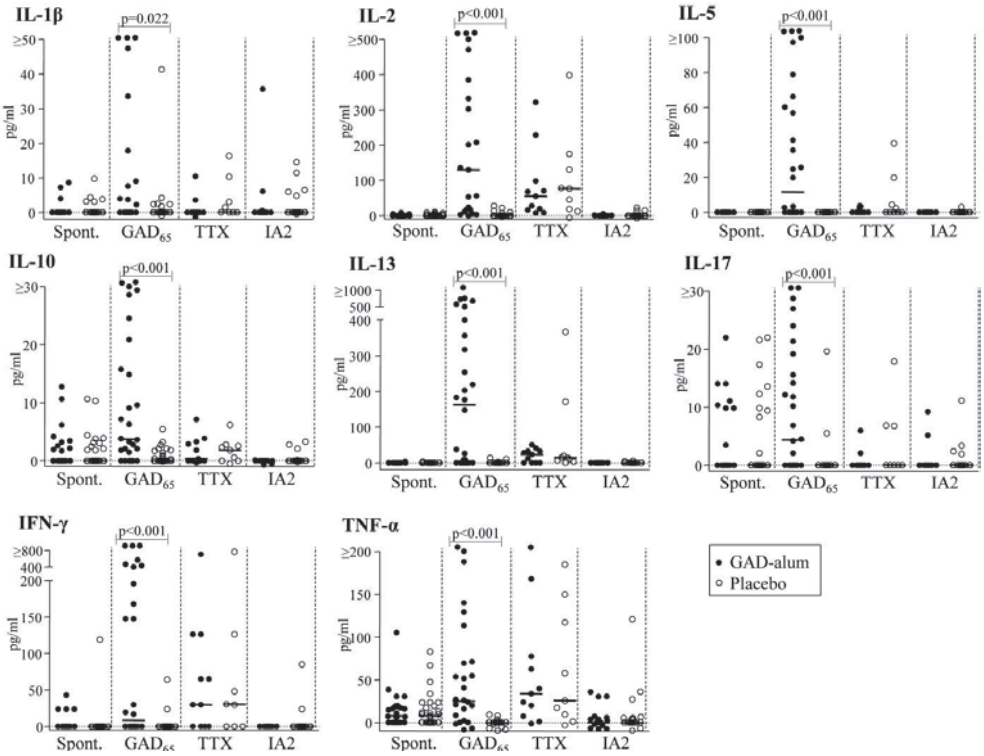

Figure 12. *Spontaneous, and GAD_{65}-, TTX- and IA-$2_{853-872}$-induced IL-1β, IL-2, IL-5, IL-10, IL-13, IL-17, IFN-γ and TNF-α secretion (pg/ml), in GAD-alum- (black circles; n=28) and placebo-treated patients (white circles; n=29), 4 years after treatment. Antigen-induced cytokine secretion is given after subtraction of spontaneous secretion. Horizontal lines represent medians.*

Taken together, these results suggest that GAD-alum treatment could exert its effect through induction of an early Th2 skewed immune response that increases over time, followed by a recall response characterized by a wide range of cytokines. These results may indicate that the immunomodulatory effect of GAD-alum is not only dependent on a predominant Th2 response, but also in several other cytokines which might be important for restoration of the immune balance.

Decreased GAD_{65}-specific Th1/Tc1 phenotype in T1D children treated with GAD-alum

We further aimed to study the immunomodulatory effect of GAD-alum treatment focusing on chemokines and chemokine receptors. Based on our results with an early Th2-associated

immune deviation in response to GAD_{65}, we hypothesized that also the GAD_{65}-induced chemokine and chemokine receptor expression might be Th2-skewed after GAD-alum treatment. Expression of Th1-associated CCR5 and Th2-associated CCR4 was analyzed on CD4+ (Th) and CD8+ (Tc) cells by flow cytometry. When stimulated with GAD_{65}, frequencies of Th1 and Tc1 cells decreased in GAD-alum-treated patients, while the placebo group remained unaffected. The amount of CCR5 present per cell was decreased following GAD_{65}-stimulation on both CD4+ and CD8+ cells in the treated group, whereas expression of CCR4 remained unaffected in samples from both groups. However, the CCR4/CCR5 ratio, indicating balance between Th2/Tc2 and Th1/Tc1 responses, increased in GAD-alum-treated patients both in CD4+ and CD8+ cells, whereas the CCR4/CCR5 ratio remained unaffected in the placebo group (Fig. 13).

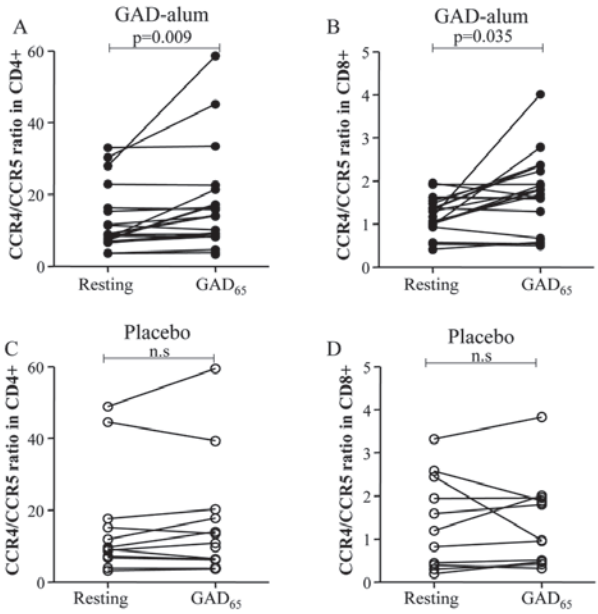

Figure 13. *The CCR4/CCR5 ratio in resting and GAD_{65}-stimulated CD4+ and CD8+ cells at 21 months after GAD-alum (black circles, n=19; A-B) or placebo (white circles, n=12; C-D) treatment. Significant differences are indicated as p-values.*

Further, secretion of the Th2-associated chemokine CCL2, a ligand to CCR4, and the Th1-associated chemokines CCL3 and CCL4, both ligands to CCR5, was analyzed in PBMC supernatants by Luminex. Upon GAD_{65}-stimulation, CCL2 increased in GAD-alum treated patients but not in the placebo group (Fig. 14), whereas secretion of CCL3 and CCL4 remained unaffected. At diabetes onset in NOD mice, CCL2 seems to correlate more closely

with an early non-destructive insulitis than with a destructive one [157-158] and primes Th2 polarization [159], whereas CCL3 secreted by antigen-specific Th1 cells invading the pancreatic islets correlates with a destructive insulitis [157]. Taken together these data suggest that the GAD-alum-induced immune responses tend to deviate away from a destructive Th1/Tc1 response upon GAD_{65} re-challenge, which may be of importance for restoring immunological balance.

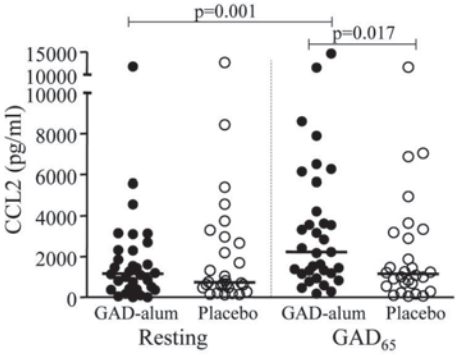

Figure 14. *CCL2 secretion (pg/ml) detected with Luminex in supernatants from resting and GAD_{65}-stimulated PBMC in GAD-alum (n=33; black circles) and placebo-treated (n=29; white circles) patients, 15 months after the first injection. Lines represent median.*

Memory responses to GAD_{65} after treatment with GAD-alum

Increased memory CD4+ T-cell frequencies after GAD_{65}-stimulation, 4 years after treatment

In 2009, a 4 year follow-up study of the clinical Phase II trial was conducted to evaluate long-term efficacy and safety of GAD-alum intervention. A significant preservation of residual insulin secretion was observed in treated patients with less than 6 months T1D duration at inclusion, compared to placebo [143]. As generation of a memory cell pool is important for an effective immune therapy, the analysis of antigen-specific memory responses may be useful to understand the duration and stability of GAD-alum-induced immune responses. Thus, we next aimed to assess long-term immune responses, 4 years after GAD-alum-treatment. The frequency of naïve (CD45RA+CCR7+), T_{CM} (CD45RA-CCR7+) and T_{EM} (CD45RA-CCR7-) cells was analyzed by flow cytometry. Our results showed that the frequency of naïve, T_{CM} and T_{EM} CD4+ cells in resting cultures did not differ between the placebo and GAD-alum groups 4 years after intervention (Fig. 15C).

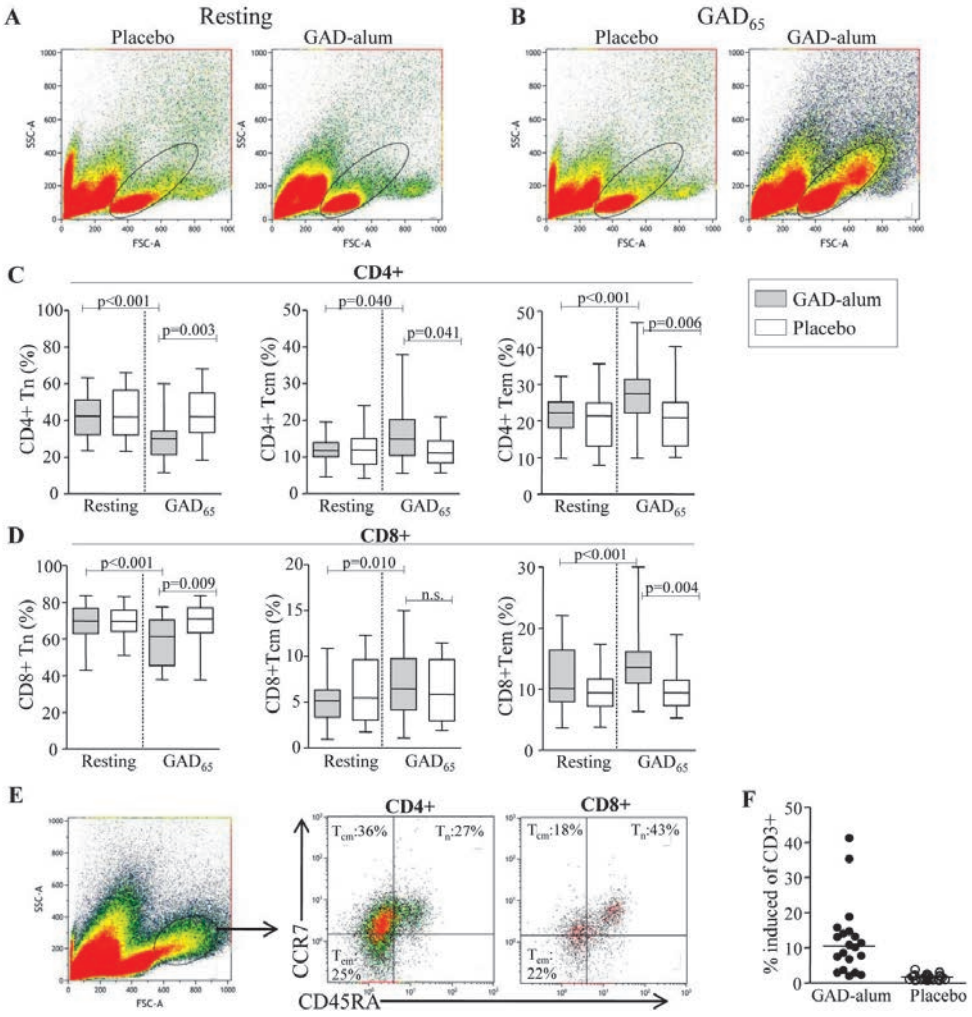

Figure 15. (A-B) *Representative flow cytometry analysis from a placebo- and a GAD-alum-treated patient assessed in resting and GAD_{65}-stimulated PBMC cultures. The gate was set to include both small and large lymphocytes.* ***(C-D)*** *Frequencies of naïve (T_n), central memory (T_{CM}) and effector memory (T_{EM}) CD4+ and CD8+ T cells, assessed in resting and GAD_{65}-stimulated PBMC cultures from GAD-alum- (n=20) and placebo-treated patients (n=23).* ***(E)*** *Representative dot plot of the GAD_{65}-induced cell subset with higher SSC and FSC evident only in GAD-alum-treated patients. Median percentages of naïve and memory CD4+ and CD8+ subsets are indicated in each plot.* ***(F)*** *Frequency of CD3+ cells that occupied the induced cell subset. Horizontal line represents median.*

In vitro stimulation with GAD_{65} induced a cell subset with higher SSC and FSC which was evident only in GAD-alum treated patients (Fig. 15E). The majority of cells within this population were CD4+ with a memory phenotype. Frequencies of both T_{CM} and T_{EM} increased whereas naïve cells decreased in GAD-alum-treated patients, while the placebo group remained unaffected (Fig. 15C). A similar pattern was also seen among CD8+ cells (Fig. 15D). This suggests a clonal expansion of the memory T-cell compartment upon antigen re-challenge, in parallel to the observed proportional reduction in naïve T-cell percentage.

In vitro stimulation with GAD_{65} induces T-cell activation 4 years after treatment

As memory T cells are characterized by a low activation threshold [73], we next analyzed the effect of antigen challenge on the induction of T-cell activation markers and proliferative responses. GAD_{65}-induced gene expression of CD69, CD25 and PD-1 was up-regulated in patients treated with GAD-alum compared to placebo, and the proliferative responses to $GAD_{65,}$ but not to control antigens, were significantly higher in treated patients (Fig. 16). These results suggest that there is a persistent GAD_{65}-specific recall cellular immune response 4 years after GAD-alum intervention.

The outcome of a T-cell response is shaped by the balance between co-stimulatory and co-inhibitory signals, which are often simultaneously provided to T cells by surrounding cells. PD-1 is a member of the CD28 superfamily of immunoreceptors that is up-regulated following TCR stimulation [160], and interaction with its ligand PD-L1 inhibits T-cell effector functions [161]. Up-regulation of PD-1/PD-L1 in parallel to GAD_{65}-induced T-cell activation and proliferation in the GAD-alum group thus demonstrates activation of co-inhibitory pathways important for regulating the immune balance.

In conclusion, persistent GAD_{65}-specific immune responses were detected 4 years after GAD-alum intervention. Prompt re-activation of GAD_{65}-reactive T cells upon in vitro antigen challenge was accompanied by induction of co-inhibitory pathways that may be of importance for regulating the GAD_{65} immunity.

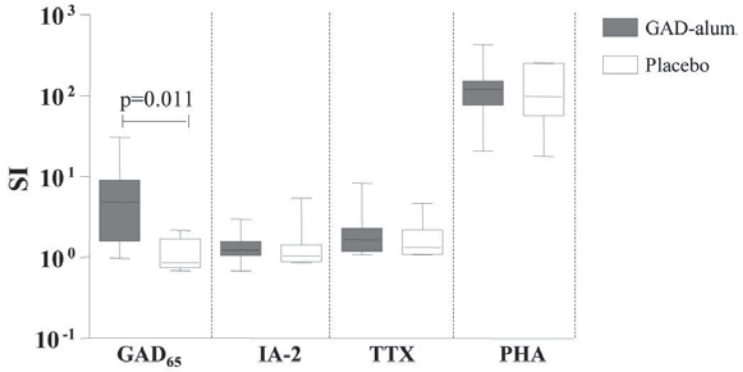

Figure 16. *Proliferative responses (median and range) to GAD_{65}, IA-2, TTX and PHA in GAD-alum- (n=10) and placebo-treated patients (n=7). Proliferation is expressed as stimulation index (SI) calculated from the mean of triplicates in the presence of stimulus divided by the mean of triplicates with medium alone.*

GAD-alum treatment enhances GAD_{65}-specific FOXP3 expression

Since one of the postulated effects of antigen immune therapy is induction of antigen-specific Treg cells able to maintain peripheral tolerance [156], we analyzed gene expression of the transcription factor FOXP3 and the cytokine TGF-β in the Phase II trial. Our results showed that the GAD_{65}-induced FOXP3 expression was higher in PBMC in the treated group compared to placebo at one, three, and nine months after intervention (Fig. 17), whereas gene expression TGF-β did not differ between the two groups. In addition, secretion of TGF-β was analyzed in the Phase III trial and in similar to the gene expression; it did not differ between the treatment arms (Fig. 10). Studies in mice suggest that therapeutic effect of anti-CD3 may involve the induction of Treg cells that can control autoimmune responses through a TGF-β–dependent mechanism [162], it is however not clear whether the same cells can be found in the circulation of T1D patients treated with anti-CD3.

Moreover, up-regulation of FOXP3 together with STAT5, which is a critical factor for inducing and maintaining the expression of FOXP3 [163], was detected upon GAD_{65}-stimulation in GAD-alum- vs. placebo-treated patients, still 4 years after treatment. A drawback in our study is that, due to limited sample volume from the children participating in the trial, analysis was limited to the PBMC pool and not in selected CD4+CD25+/CD4+ CD25- T cell populations.

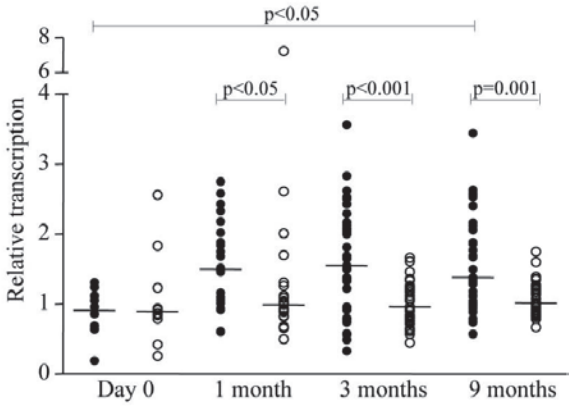

Figure 17. *GAD_{65}-induced FOXP3 mRNA detected by RT-PCR in PBMC at baseline (Day 0) and one, three and nine months after receiving GAD-alum (black circles) or placebo (white circles). FOXP3 mRNA levels are expressed as relative transcription based on the calculation $2^{-\Delta\Delta Ct}$. Horizontal lines represent the median.*

These results support the presence of a GAD_{65}-specific cell population in the periphery with regulatory properties which is in line with our previous findings [151].

The effect of GAD-alum is antigen-specific

To confirm that the effect of GAD-alum was antigen-specific, cytokine secretion and PBMC proliferative response was analyzed to several control antigens including TTX and IA-2. *In vitro* stimulation with antigens other than GAD_{65} did not enhance cytokine secretion (Fig. 12) or PBMC proliferation (Fig. 16) in the treated group compared to placebo, which supports the antigen-specific effect of GAD-alum. This selective immune modulation might be seen as an indication for safety, since it would be undesirable to non-specifically influence immune responses to unrelated antigens. Immune responses to control antigens have also been demonstrated to be unaffected by Diapep277 treatment [149] as well as nasal insulin [150].

Moreover, spontaneous cytokine and chemokine secretion, i.e. cell cultures without stimulus, did not differ between GAD-alum-treated and placebo patients at any time point.

Cytokine secretion and β-cell function

Reliable biomarkers associated with therapeutic success following interventions with β-cell antigens are still lacking. In order to search for immune surrogate markers for clinical efficacy, we analyzed the association between cytokine and chemokine secretion and β-cell function, measured by stimulated C-peptide. Our results from the Phase II study show that early after treatment, GAD_{65}-induced IL-13 was higher while IL-5 tended to be higher in the small group of patients classified as clinical responders compared with non-responders (Fig. 18), with the highest levels observed in the three patients with loss of stimulated C-peptide less than 10 % over the first 15 months after treatment. Although encouraging, we have to keep in mind that these finding is based on a small number of patients.

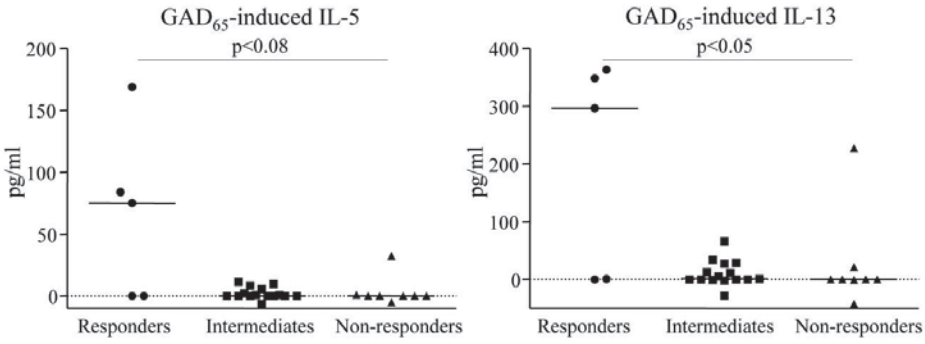

Figure 18. *GAD_{65}-induced IL-5 and IL-13 secretion, one month after treatment. The GAD-alum treated patients were stratified into three groups; Responders (loss of AUC < 10 %, n=5), Intermediate responders (loss of AUC between 10 % and 65 %, n=15) and Non-responders (loss of AUC > 65 %, n=8). Three responder patients showed the highest levels of both IL-5 and IL-13. GAD_{65}-induced cytokine levels are given after subtraction of spontaneous responses and expressed as pg/ml. Horizontal lines represent the median.*

An association of Th2 cytokine secretion and clinical effect was however not confirmed in the Phase III trial, where we instead found that a predominant secretion of pro-inflammatory cytokines early after GAD-alum treatment was associated with low stimulated C-peptide at 30 months. These results together support a shift in GAD_{65}-specific immune responses from a destructive Th1 to a protective or non-pathogenic Th2 response. We also demonstrate that even if additional doses of GAD-alum resulted in a stronger immune response, the phenotype of the immune response was similar in the 2D and 4D regimens (Fig. 11), and this stronger immune response was not related to clinical outcome.

The relative contribution of each chemokine to the GAD_{65}-induced secretion shows that responders and intermediate responders to the treatment in the Phase II trial were characterized by a predominant Th2-associated profile, whereas non-responder patients display a more pronounced Th1-associated chemokine secretion (Fig. 19). This is in line with our cytokine data which further supports the hypothesis that a protective effect could be induced by a shift from Th1 to Th2 in response to GAD_{65}.

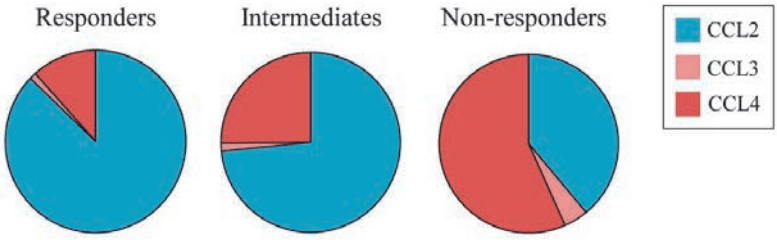

Figure 19. *Pie charts illustrating the relative contribution of each of the chemokines CCL2, CCL3 and CCL4 to the GAD_{65}-induced secretion at 21 months detected by Luminex in Responders (n=7), Intermediates (n=14) and Non-responders (n=7) to GAD-alum treatment.*

Moreover, 4 years after treatment the cytokine profile in patients with a loss of stimulated C-peptide AUC ≤ 60 % was characterized by a more pronounced GAD_{65}-induced Th2/Treg associated secretion, whereas patients with a loss of C-peptide AUC > 60 % had a more pronounced pro-inflammatory profile secretion (Fig. 20). Even though not statistically assured, this may suggest that a beneficial clinical response might be associated with a persistent Th2/Treg-skewed GAD_{65}-specific immune response, which also is consistent with our previous findings.

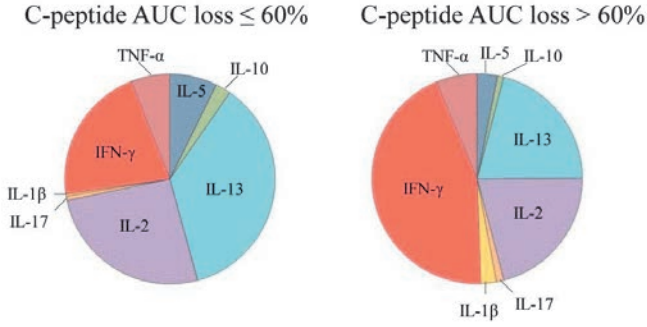

Figure 20*. Cytokine profiles in GAD-alum patients with a loss of C-peptide AUC < 60% (n=5), or a loss of C-peptide AUC > 60% (n=16). Pie chart illustrates the relative contribution of each cytokine to the GAD_{65}-induced secretion detected by Luminex.*

Together these findings suggest that a beneficial clinical response to GAD-alum treatment might be associated with a persistent Th2/Treg associated GAD_{65}-specific cytokine and chemokine response. Moreover, since two additional injections of GAD-alum did not show clinical benefit, our results suggests that the intensity of the immune response after antigen administration does not necessarily have to be related to therapeutic efficacy.

Preserved C-peptide 30 months after GAD-alum treatment in recent-onset T1D children

We have shown preservation of residual insulin secretion by GAD-alum treatment in a Phase II trial involving recent-onset T1D children [142-143]. In the Phase III trial, 2 additional doses of GAD-alum were given in one of the treatment arms to test if this would improve the clinical effect. Unfortunately treatment with GAD-alum did not significantly reduce the loss of stimulated C-peptide or improve clinical outcomes over a 15-month period in any of the treatment arms [145]. The trial was therefore closed after 15 months, and the 30 months follow-up period was completed only by a minority of the patients. Since patients in Sweden entered the trial relatively early compared to the rest of the European countries, a majority of the Swedish patients completed their 21 months visit, and some also completed the 30 months follow-up.

Comparison of fasting and stimulated C-peptide secretion at 21 months did not reveal any differences between the three treatment arms (Fig. 21A-B). However, analysis of the subgroup of 45 patients that completed the 30 months visit, showed a reduced loss in fasting and meal stimulated C-peptide AUC at 30 months in the 2D regimen compared to placebo (Fig. 21C-D). While fasting and stimulated C-peptide levels from patients in the placebo and 4D regimens continued to decline between 21 and 30 months visit, patients from the 2D group did not lose further C-peptide.

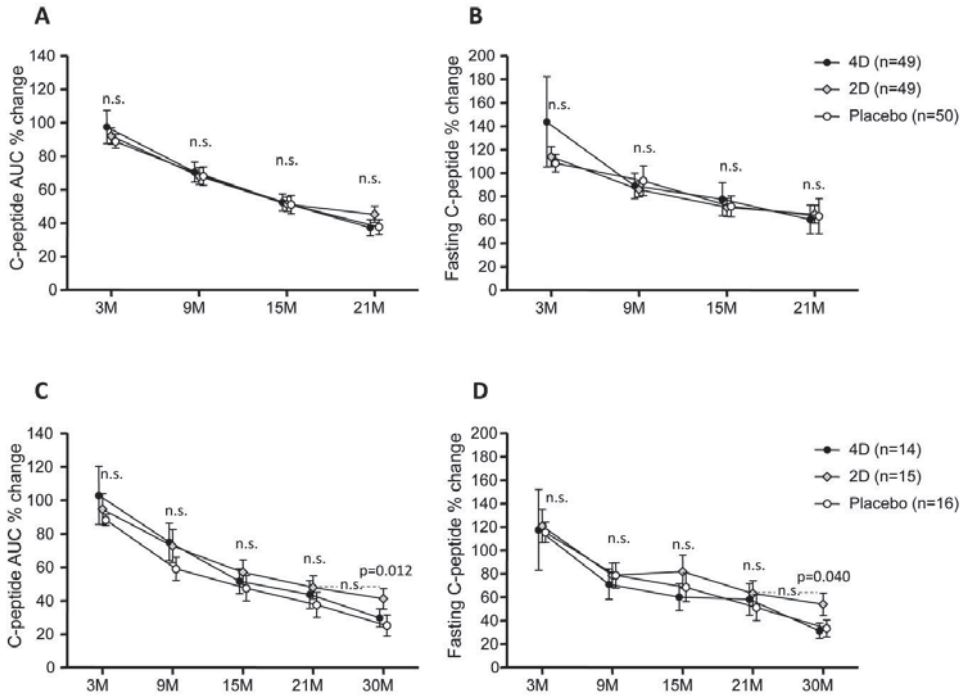

Figure 21. *Changes in stimulated and fasting C-peptide (mean±SEM), reported as a percentual change from baseline, in patients receiving 4 doses of GAD-alum (4D, black circles), 2 doses of GAD-alum (2D, gray rhombuses) or placebo (white circles), in the entire Swedish cohort up to 21 months* ***(A-B)*** *and for the Swedish subgroup of patients who completed the 30 month visit* ***(C-D)****. Significant differences are indicated as p-values*.

In addition, significantly more patients in the 2D regimen preserved more than 25 % of their initial stimulated C-peptide AUC compared to patients in the placebo arm after 30 months (Fig. 22A). Also, the proportion of patients with peak C-peptide > 0.2 nmol/l after meal stimulation, tended to be larger in the 2D regimen than in the placebo group after 30 months (Fig. 22B). These results are encouraging, since even a modest residual insulin secretion, with stimulated C-peptide levels > 0.2 nmol/l has been reported to provide clinically meaningful benefits in terms of reducing long-term complications [29].

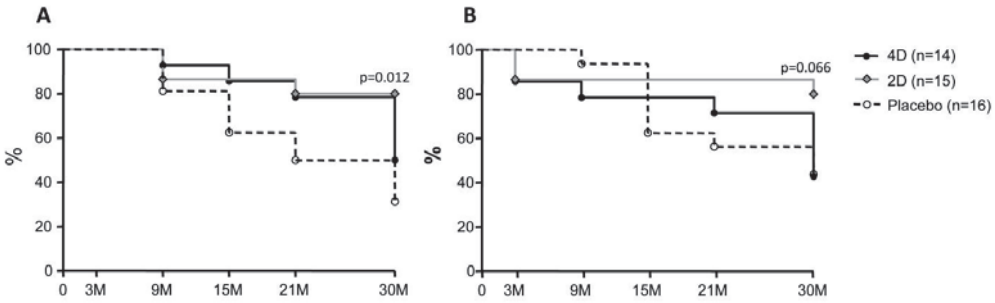

Figure 22. *(**A**) The proportion of patients (%) preserving > 25 % of their initial C-peptide AUC from baseline to 30 months, and (**B**) the proportion of patients achieving a peak stimulated C-peptide level > 0.2 nmol/l from baseline to 30 months after treatment with 4 doses of GAD-alum (4D, black circles), 2 doses of GAD-alum (2D, grey rhombuses), or placebo (white circles).*

The reason why clinical effect of GAD-alum was not detected until 30 months after treatment might be due to the rather small loss of C-peptide in diabetic teenagers during the first 12-15 months after diagnosis, as seen in a Swedish national cohort of approximately 4000 newly-diagnosed children and adolescents with T1D (Ludvigsson *et al* to be published), and also seen in a large European cohort of patients with T1D [164].

Finally, to confirm that the small subgroup of patients followed for 30 months represent the entire study cohort, fasting and stimulated C-peptide in the 2D regimen was compared to the Swedish cohort and the entire European study cohort. The small subgroup did not significantly differ from the other cohorts at any time point; furthermore there were no differences in patient's baseline characteristics.

Taken together these results suggest that 2 doses of GAD-alum did have an effect on preservation of residual insulin secretion, but the effect was not apparent until after 30 months.

CONCLUDING REMARKS

Clinical intervention trials in recently diagnosed T1D patients are important since even modest preservation of β-cell function is recognized to result in reduced end-organ complications. In this thesis I report findings from our immunological studies within two clinical trials in T1D children with GAD-alum. In order to search for immune surrogate markers of clinical efficacy, we analyzed the association between cytokine secretion, chemokine/chemokine receptor expression and β-cell function. Taken together, our results suggests that GAD-alum treatment could exert its effect through induction of an early Th2 skewed immune response which tend to deviate away from a destructive Th1/Tc1 response upon GAD_{65} re-challenge. We also studied long-term responses, since the analysis of antigen-specific memory responses may be useful to understand the duration and stability of GAD-alum-induced immune responses. Our results showed persistent GAD_{65}-specific immune responses 4 years after GAD-alum intervention. Prompt re-activation of GAD_{65}-reactive T cells upon *in vitro* antigen challenge was accompanied by secretion of Th1, Th2 and Treg cytokines and by induction of co-inhibitory pathways that may be of importance for regulating the GAD_{65} immunity.

Our findings provide new insights to the understanding of the immunomodulatory effects by antigen-specific immunotherapy in T1D. The usage of Luminex and PCR array is a huge advantage in our projects within the GAD-alum trials, since these methods enable evaluation of multiple analytes simultaneously, which is of great value in situations where sample volume is limiting. We have consequently been able to study a larger network of cytokines which is in contrast to several previous immunomodulatory studies in both mice and humans, where only a few cytokines have been analyzed. However, a clear effect of immunomodulatory therapies on disease mechanisms remains uncertain, in part because studies are limited to the analysis of peripheral blood which may not be the major site of action of the immunomodulatory agents. Much remains to be clarified regarding antigen therapy as an approach to prevent β-cell destruction. Thus, continued research to better understand how immunomodulation with autoantigen modifies T- and B-cell responses is crucial to optimize future intervention trials using β-cell antigens.

It is possible that using different doses, different frequency of administration, different adjuvants or perhaps different routes of administration would improve the effect. Yet another

approach is administration of GAD-alum treatment prior to the clinical onset, at a time when β-cell mass is more substantial than at diagnosis. The ongoing prevention trial DiAPREV-IT will hopefully be able to answer this in the near future. Furthermore, the lack of permanent remission of T1D with any single agent suggests that future studies may include investigation of GAD-alum in combination with other therapies.

In parallel to this thesis, several other projects involving Treg cell frequency, phenotype and function as well as autoantibody levels and IgG subclass distribution have been performed or are currently ongoing within the Phase II and III GAD-alum trials. When we started, only one previous trial had tested GAD-alum in LADA patients, thus our hypotheses were mainly based on murine studies. Hopefully, our results all together will increase the knowledge of immunomodulatory effects after antigen treatment in humans, and bring the T1D research field further in the quest of finding successful intervention strategies.

Since the Phase III study failed to reach its primary outcome, a main question is why the efficacy differed from the previous Phase II study. We plan to perform a 4 year follow-up study of patients included in the Phase III trial and analyze their residual insulin secretion which hopefully will confirm the clinical benefit that was observed in the subgroup of patients 30 months after GAD-alum treatment. This study could potentially give further valuable proof of GAD-alum as an immunomodulator in T1D.

Moreover, widespread influenza A (H1N1) vaccinations coincided with the Phase III study period, and the vast majority in the Swedish cohort received influenza vaccination. Thus it will be important to assess whether this vaccination induced immune responses that interfered with the effect of GAD-alum treatment. A future goal will also be to clarify whether heterogeneous etiology of the disease or differences in the autoimmune response is a possible explanation for the variable outcome between the Phase II and Phase III trial. Yet, continued research to find biomarkers that identify patients that respond to immune interventions with β-cell antigens, will also be an essential goal in the future.

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