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REIGN IN BLOOD
IMMUNE REGULATION IN TYPE 1 DIABETES

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“Just leave me alone, I know what I’m doing.”

Kimi Räikkönen

on team radio while leading the 2012 Abu Dhabi Grand Prix

ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune disease resulting in insulin deficiency as a result of autoimmune destruction of pancreatic β-cells. Preserving β-cell function in patients with T1D would be of great benefit since patients with sustained endogenous insulin secretion are known to suffer less from secondary complications due to hyperglycemia. Glutamic acid decarboxylase 65 (GAD_{65}) is a major autoantigen targeted by self-reactive lymphocytes in T1D, and has been used in several attempts at treating T1D by inducing tolerance to β-cell antigens. We showed positive clinical effects of GAD_{65} formulated with aluminium hydroxide (GAD-alum) on preservation of C-peptide secretion in a phase II clinical trial. Unfortunately, a phase III clinical trial in a larger population failed to confirm this finding. Regulatory T cells (Treg) are instrumental in maintaining peripheral tolerance to self-antigens. Deficiencies in Treg function are thought to influence the pathogenesis of autoimmune diseases, including T1D. One proposed mechanism of achieving tolerance to β-cell antigens in T1D is the induction of antigen-specific Treg through immunomodulation. The general aim of this thesis was to study immune regulation in T1D, the role of Treg and immunomodulatory effects of GAD-alum treatment in particular. Our hypothesis was that Treg biology is altered in T1D and pre-diabetes, and that an induction of GAD_{65}-specific Treg contributes to the clinical efficacy of GAD-alum treatment. We demonstrated that T cells expressing Treg-associated markers were increased in number in patients with recent-onset T1D, as well as in children with high risk of developing T1D. We found that antigen recall 4 years after GAD-alum treatment induced cells with both regulatory and effector phenotypes in GAD-alum treated patients. Furthermore there was no effect on Treg-mediated suppression in GAD-alum treated patients, while patients with T1D, regardless of treatment, exhibited deficient Treg-mediated suppression of Teff that was intrinsic to the Treg population. We followed patients participating in a phase III trial of GAD-alum, and using an extended antibody panel we demonstrated that antigen recall induced mainly Teff cells in treated patients, along with increased frequencies of memory T cells, non-suppressive $CD45RA^{-}FOXP3^{lo}$ cells and increased GAD_{65}-induced proliferation of mainly Teff and memory T cells. Finally we examined whether SNPs in genes encoding inflammasome components contributed to T1D risk, but found no effects of variant alleles on the risk of developing T1D, or on the efficacy of GAD-alum treatment. We show small effects on C-peptide secretion and autoantibody positivity in patients with T1D. In conclusion, we find that while Treg are deficient in patients with T1D, induction of Treg is an unlikely mechanism of action of GAD-alum treatment.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Typ 1 Diabetes (T1D) eller barndiabetes är en autoimmun sjukdom där kroppens eget immunförsvar attackerar bukspottkörtelns insulinproducerande β-celler, vilket resulterar i insulinbrist och därmed kroniskt förhöjt blodsocker. Sverige har världens näst högsta förekomst av T1D efter Finland, och antalet nyinsjuknande har ökat världen över de senaste decennierna av oklara skäl. Vad som orsakar sjukdomen är också okänt. Hos patienter med T1D reagerar immunförsvaret på insulin och andra ämnen som produceras i β-cellerna, bland andra glutaminsyra dekarboxylas (GAD_{65}). Vi har visat att ett sorts diabetesvaccin bestående av GAD_{65} kopplat till aluminium hydroxid (GAD-alum) bevarade β-cellsfunktionen hos nyinsjuknade barn med T1D. En bevarad insulinproduktion är ytterst önskvärd då patienter som kan producera eget insulin har lättare att kontrollera sitt blodsocker och mer sällan drabbas av både akuta komplikationer och komplikationer senare i livet. Den kliniska effekten av GAD-alum åtföljdes av immunologiska förändringar vilka skulle kunna förklara den kliniska effekten. Regulatoriska T celler (Treg) spelar en central roll i upprätthållandet av tolerans mot kroppens egna vävnader, och man har visat bristande funktion hos Treg i flera autoimmuna sjukdomar, inklusive T1D. Vår hypotes är att GAD-alum behandling inducerar antigenspecifika Treg mot GAD_{65} som bidrar till en ökad tolerans och en minskad immunmedierad attack mot bukspottkörteln.

Syftet med avhandlingsarbetet var att studera immunreglering i T1D, framförallt Treg-medierad reglering, samt att studera effekten av GAD-alum behandling på immunreglering vid T1D för att öka kunskapen om immunologiska mekanismer bakom immunmodulerande behandling av T1D. Våra resultat visar att barn med T1D har högre nivåer av celler som uttrycker Treg-markörer, men deras Treg är sämre på att trycka ner immunologiska reaktioner hos andra T celler som tros förorsaka sjukdomen. Vidare visar vi att medan barn som behandlats med GAD-alum svarar på antigenstimulering med högre antal GAD_{65}-specifika celler, så ökar både Treg och aktiverade T celler, dock främst icke-regulatoriska samt minnes T celler. Fyra år efter GAD-alum behandling kunde vi inte påvisa någon effekt på Treg-medierad nedreglering av andra T celler. Vi har även visat att mutationer i gener som kodar för beståndsdelar i ett proteinkomplex kallat inflammasomen som skulle kunna bidra till autoimmunitet inte påverkar risken att insjukna i T1D. Mutationerna påverkade inte heller den kliniska effekten av GAD-alum behandling, men patienter med mutationer hade något sämre insulinproduktion.

Sammanfattningsvis pekar våra resultat på att GAD-alums kliniska effekt är kopplad till GAD_{65}-specifika immunologiska förändringar, men effekten verkar inte åstadkommas genom effekter på antalet Treg eller deras funktion. Däremot visar vi en bristande Treg-funktion hos patienter med T1D, medan inflammasomen inte verkar spela någon större roll för sjukdomsförloppet eller effekten av GAD-alum behandling.

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

Pihl M, Chéramy M, Mjösberg J, Ludvigsson J, Casas R

Increased expression of regulatory T cell-associated markers in recent-onset diabetic children
Open Journal of Immunology, 2011; 1(3):57-64

Paper II

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

Regulatory T cell phenotype and function 4 years after GAD-Alum treatment in children with Type 1 Diabetes
Clinical and Experimental Immunology, 2013; 172(3):394-402

Paper III

Pihl M, Axelsson S, Chéramy M, Reijonen H, Ludvigsson J, Casas R

GAD-alum treatment induces GAD-specific CD4 T cells in a phase III clinical trial
Manuscript

Paper IV

Pihl M, Verma D, Söderström M, Söderkvist P, Ludvigsson J, Casas R

Polymorphisms in NLRP3 inflammasome components NLRP3 and CARD8 affect C-peptide secretion in type 1 diabetes
Manuscript

ABBREVIATIONS

APC	Antigen presenting cell
ATP	Adenosine triphosphate
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CARD8	Caspase recruitment domain-containing protein 8
CD	Cluster of differentiation
C-peptide	Connecting peptide
C_t	Threshold cycle
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
FOXP3	Forkhead box P3
FSC	Forward scatter
GABA	γ-aminobutyric acid
GAD_{65}	Glutamic acid decarboxylase
GADA	Glutamic acid decarboxylase autoantibodies
GAD-alum	Aluminium hydroxide formulated GAD_{65}
GITR	Glucocorticoid-induced TNFR-related protein
HLA	Human leukocyte antigen
IA-2	Insulinoma-associated antigen 4
IFN	Interferon
IL	Interleukin
LADA	Latent autoimmune diabetes in adults
MFI	Median fluorescence intensity
NALP3	NACHT, LRR and PYD domains-containing protein 3
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SSC	Side scatter
T1D	Type 1 Diabetes
TCR	T cell receptor
Teff	Effector T cell
TGF	Transforming growth factor
Th	T helper
TMR	Tetramer
TNF	Tumor necrosis factor
Treg	Regulatory T cell
ZnT8	Zinc transporter 8

INTRODUCTION

Type 1 Diabetes

Definition and diagnosis

Diabetes Mellitus is a group of metabolic diseases resulting in hyperglycemia as a consequence of insulin deficiency, resulting either from inadequate insulin production or inadequate insulin effect on target tissue [1]. Diabetes is divided into two main categories, Type 1 and Type 2 Diabetes, where Type 1 Diabetes (T1D) is caused by an absolute deficiency of insulin secretion [2]. T1D has previously been known as insulin-dependent diabetes mellitus or juvenile-onset diabetes. Common symptoms preceding diagnosis are increased thirst and urination in combination with weight loss. Diagnosis is made based on fasting plasma glucose or based on glucose measurement following an oral glucose tolerance test. The criteria for diagnosis are fasting plasma glucose >7.0 mmol/l, or >11.1 mmol/l postload glucose, or symptoms of hyperglycemia and casual plasma glucose >11.1 mmol/l, according to the American Diabetes Association [2]. Glucose testing is carried out according to guidelines established by the World Health Organization [1]. Patients with T1D require multiple injections of exogenous insulin to maintain normal blood glucose levels, which is particularly difficult for very young children to cope with in the long term.

Incidence

Second to Finland, Sweden has the highest incidence of T1D in the world, at 30 cases / 100.000 / year in children under 14 years of age in the ‘80s and ‘90s [3]. The incidence of T1D has been increasing during the last decades, from 25,8 cases /100.000 / year among children born between 1989-1993 to 34,6 /100.000 / year among children born in 1999-2003 [4], and in recent years 40-45/100.000/year [5]. Age at onset tended to decrease in Swedish patients born in the early 90s, with a higher proportion of children below the age of 6 developing T1D [6]. However, both these trends may have leveled off or reversed in Swedish children born after the year 2000 [7].

Pathogenesis

The etiology of T1D is considered a complex interplay of genetic and environmental risk factors and life style. The disease is mediated by an autoimmune process resulting in destruction of insulin producing pancreatic β-cells. At or shortly after diagnosis, most islets are deficient in β-cells, which is also true for patients with long-standing T1D. Islets sometimes contain cells with enlarged nuclei, and an infiltrate of cells referred to as insulitis [8]. Hyperexpression of human leukocyte antigen (HLA) class 1 on the remaining islet cells is also common, which might affect β-cell destruction by $CD8^+$ cytotoxic T cells. The majority of cells in the inflammatory infiltrate are $CD8^+$ cytotoxic T cells, but it is also composed of $CD4^+$ T helper (Th) cells, macrophages, B cells and natural killer cells. Autoimmune destruction of the islets may be ongoing long before clinical symptoms manifest. Evidence indicates that approximately 80% of the β-cell mass can be lost before symptoms appear (Fig 1) [9]. It is thought that the metabolic activity of individual islets affect the rate at which they are destroyed. A phenomenon known as the honeymoon period or partial remission occurs in some patients in conjunction with initiation of insulin treatment after diagnosis. It occurs because not all of the pancreatic β-cells are destroyed and viable cells produce unpredictable amounts of endogenous insulin when the excess blood glucose is removed by exogenous insulin, producing effects resembling remission. Furthermore, improved metabolism is associated with improved insulin sensitivity. The partial remission varies from patient to patient, and may last for weeks or months, or in older children and adolescents even years.

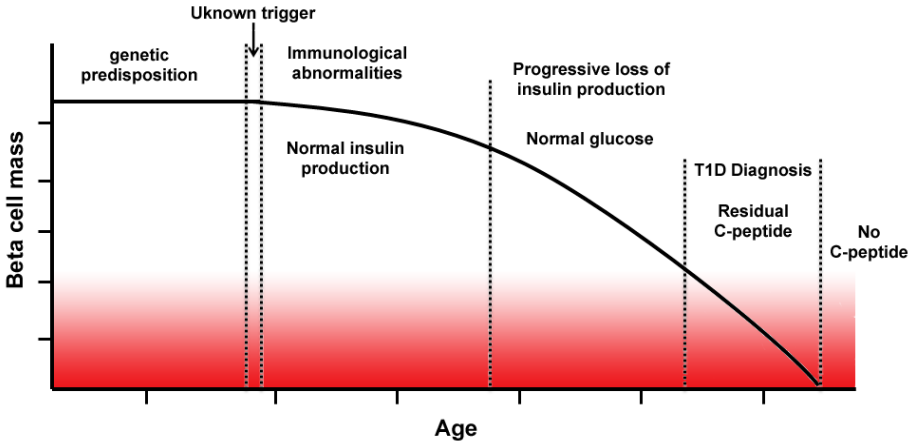

Figure 1. *Model of β-cell destruction over time, preceding and after clinical onset of T1D. Based on the model proposed in [10].*

Treatment

Despite several decades of intense research, there is still no cure for T1D. Treatment consists of insulin administration by means of injections or through continuous delivery using pumps. Insulin treatment gives patients with T1D considerably better quality of life and life expectancy than in the past. Although modern insulin treatment enables reasonably tight control of blood glucose, which reduces the risk of diabetes-related complications like retinopathy, neuropathy, cardiovascular complications and nephropathy, many patients still eventually suffer from such secondary complications, leading to reduced vision or rarely even blindness, cardiovascular disease, renal failure and increased mortality [11-13].

Insulin

Insulin is produced from proinsulin by cleaving a connecting peptide (C-peptide) from proinsulin. C-peptide facilitates proper folding of the protein prior to cleavage (Fig 2). C-peptide is released together with insulin at equimolar concentration. Contrary to insulin, C-peptide does not undergo hepatic extraction and thus has a considerably longer plasma half life, about 30 minutes compared to 3-4 minutes for insulin. For this reason, and especially as C-peptide in contrast to insulin is not injected, C-peptide is used to monitor insulin secretion in clinical assays [14].

During the last decade several studies have suggested that C-peptide is a bioactive peptide on its own, with important effects on vascular endothelial function. It was thus hypothesized that decreased C-peptide levels in patients with T1D could contribute to secondary complications, and eventually shown that co-administration of C-peptide with insulin to patients with T1D reduces renal and nerve dysfunction [15]. There is also indication that C-peptide binds insulin in plasma which affects its biological availability, resulting in better utilization of glucose.

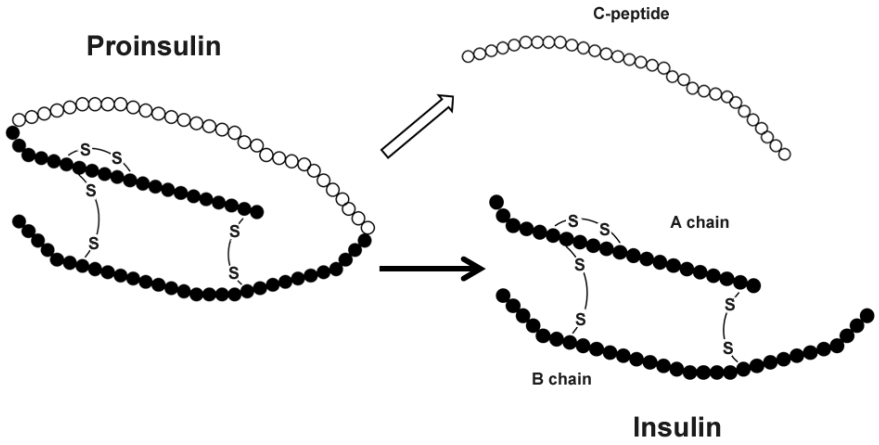

Figure 2. *Schematic description of formation of mature insulin and C-peptide through proteolytic cleavage of proinsulin.*

Risk factors

Genetic risk factors

Studies of monozygotic twins have found a high concordance rate of T1D compared to dizygotic twins and siblings [16], indicating a strong genetic component in T1D risk but also implying that environmental risk plays a significant role. Reported concordance rates among monozygotic twins vary between 21 and 70%. A recent study found a cumulative incidence of 65% at 60 years of age, while 78% of twins to patients with T1D were persistently positive for autoantibodies [17]. Genes in the highly polymorphic HLA class II gene complex, which encodes molecules responsible for presenting antigen to T lymphocytes, are the most dominant in conferring genetic risk for T1D, accounting for roughly half the genetic

component [18]. The association with the HLA region is complex, the main determinant being heterodimers of HLA-DQA1 and –DQB1, while HLA-DR molecules have a modifying effect on disease risk. Combinations of DR4-DQ8, DR3-DQ2 and DR4-DQ2 molecules are considered to confer higher risk of developing T1D whereas for example DR4-DQ7 is considered protective.

The remaining half of genetic risk is not yet completely accounted for, but is in part determined by polymorphisms in a large number of genes. Among them are the genes encoding insulin, Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), Interleukin (IL)-2 receptor alpha chain and protein tyrosine phosphatase N22 [19-20]. The disease-associated polymorphism in the Insulin gene has been shown to affect the number of tandem repeats in the promoter region of the gene, affecting its expression in the thymus and thus the deletion of autoreactive T cells [21]. Taken together, the genes conferring risk for T1D are involved in regulation of the immune system; the HLA genes and insulin affect the presentation of a common autoantigen in T1D, while IL-2Rα/CD25 and CTLA-4 are both involved in T cell homeostasis and activation, as well as regulatory T cell (Treg) homeostasis and function. This is very much consistent with T1D being an autoimmune disease, and the number of genes involved in T1D pathogenesis highlights the complex interplay in which multiple pathways may be essential for developing autoimmunity. Very high and high risk HLA alleles are more prevalent among Swedish children with T1D, while moderate and low risk alleles are more common among immigrants with T1D in Sweden, illustrating differences in genetic risk depending on ethnicity [22].

Environmental risk factors

Interestingly, children born in Sweden to immigrants from low-risk regions like East Asia have higher incidence of T1D compared to adopted children and immigrants from low-risk regions [23]. This indicates that exposure to environmental factors *in utero* or during infancy play a substantial role in the pathogenesis of T1D. The risk of developing T1D among children born in Sweden to immigrants is considerably lower compared to children born to Swedish parents, however. According to the hygiene hypothesis, commonly discussed in relation to atopic disease, exposure to certain infectious agents at a young age affects the immune system and decreases the risk of atopic disease. Similar mechanisms have been implicated in settings of autoimmune disease [24]. In a study of adopted children with T1D in

Sweden however, the incidence of T1D did not vary with age at adoption, suggesting that the hygiene hypothesis might not be as relevant for T1D as for atopic disease [23].

Other environmental factors that may affect T1D risk are short breastfeeding, early introduction of cow's milk, gut microflora and infections with enteroviruses [25-26], while vitamin D supplementation during infancy and long breastfeeding has a protective effect [27-28]. Bovine insulin, present in cow's milk, differs from human insulin by only three amino acids and can be considered a modified self-antigen, as such it may escape tolerance to self-insulin established in the thymus, resulting in autoimmunity to endogenous insulin. Introduction of cow's milk in the diet before the age of 3 months has been shown to affect insulin reactivity [29]. This was later shown to be influenced by enterovirus infection, resulting in increased levels of antibodies against bovine insulin [30].

Evidence suggest that viral infections, particularly Coxsackie B infection, can damage β-cells both directly through β-cell infection or indirectly through induction of proinflammatory signaling [31]. If endogenous antigens are released as a consequence of tissue damage in the presence of proinflammatory mediators, it could facilitate activation of self-reactive T cells, a process called bystander activation. Antibodies against Coxsackie B virus, indicating a previous infection, are associated with increased risk of T1D development [32]. There is potential molecular mimicry between parts of the P2-C protein expressed by Coxsackie B virus and peptides within the 247-279 amino acid region of human glutamic acid decarboxylase (GAD_{65}), which could explain mechanistically how infection leads to autoimmunity [33]. T1D diagnoses peak during autumn and winter and decreases during summer [34], and while viral infections have been suggested to account for this seasonality of disease onset, a link between infection and seasonality has not been confirmed [35]. Congenital rubella and maternal enterovirus infections during pregnancy have also been associated with risk for T1D [36-37].

It has been suggested that β-cell stress may be a risk factor for T1D development [38]. The demand for insulin production increases during periods of rapid growth, for example during puberty, and this increased production of insulin may result in increased β-cell stress and consequently provide stimulation for initiation of an autoimmune process.

Immunology of T1D

T1D as an autoimmune disease

There is extensive evidence that T1D is an autoimmune disease, but this remains to be strictly proven. The previously mentioned inflammatory infiltrate is one indicator that the disease is immunologically mediated. Most of the genes conferring risk for T1D are also involved in immunity. Furthermore, antibodies reactive to insulin [39], GAD_{65} [40], zinc T8 transporter (ZnT8) [41] and insulinoma-associated antigen-2 (IA-2, also known as tyrosine phosphatase islet antigen 2) [42] are often present in patients with T1D. T1D can be adoptively transferred between mice by both $CD4^+$ and $CD8^+$ T cells [43-44]. One group considers their recent finding of islet-autoreactive $CD8^+$ T cells in insulitic lesions definitive evidence that T1D is an autoimmune disease [45], but this interpretation is not consensus within the scientific community. Some argue that T1D could be regarded as an innate autoinflammatory disease that could be caused by infection-induced inflammation [46]. In addition, autoreactive T cells recognizing insulin and GAD_{65} are present in healthy individuals to the same extent as in patients with T1D [47-48], though the GAD_{65}-specific T cells are exclusively naïve in healthy subjects whereas GAD_{65}-specific T cells are also found within the memory T cell pool in patients with T1D [48]. The same pattern of naïve and memory T cells in healthy individuals and patients with T1D has been observed in both $CD4^+$ and $CD8^+$ T cells recognizing GAD_{65} and insulin peptides [49].

Antigens and antibodies

There are four major established autoantigens against which autoantibodies are formed that are used to predict development of T1D; GAD_{65}, IA-2, insulin and ZnT8 [41]. All the proteins targeted as autoantigens are potentially secreted by the β-cell, but apart from insulin their function in the β-cell is not completely determined. Before antibodies to specific antigens were identified, scientists tested sera from patients against samples of pancreatic tissue to detect islet cell antibodies (ICA), but the assay does not reveal the specificity of the antibodies [50].

GAD was identified as an autoantigen in T1D after it had been shown that patients recently diagnosed with T1D commonly displayed immune reactivity against the then unknown protein [40,51]. It was later discovered that GAD is an enzyme that produces a neurotransmitter called γ-amino butyric acid (GABA) from glutamic acid through

decarboxylation. Two isoforms of the enzyme exist with 65% homology in their amino acid sequence, GAD_{65} and GAD_{67}, with different molecular weight but identical enzymatic activity [52]. Both isoforms are expressed in the CNS, while only GAD_{65} is expressed in β-cells where it localizes to secretory vesicles. GABA is however not released together with insulin from the β-cell, but is contained in separate vesicles. It has been suggested that β-cell derived GABA is involved in regulation of secretion of insulin, somatostatin and glucagon [53].

IA-2 is a transmembrane protein located in secretory granules of neuroendocrine cells. Its function in islet cells is unknown but it has been suggested to be involved in regulating the content of secretory granules and β-cell growth [54]. ZnT8 was identified as an autoantigen in T1D fairly recently, and is one of several zinc transporters expressed by β-cells [41]. Its exact function is still unknown, but it is associated with the regulated pathway of secretion and might contribute to the concentration of Zn^{2+} in the granules [55].

An estimated 98% of all patients are positive for one or more of these autoantibodies at clinical onset of T1D. Individuals who produce ICA and two or more additional autoantibodies have greater than 60% risk of developing T1D within 5 years [56]. First degree relatives of patients with T1D who test positive for two or more autoantibodies against GAD_{65}, insulin, IA-2 reportedly have a 68% 5 year risk of developing T1D, while the 5 year risk of first degree relatives positive for all three autoantibodies was estimated to be 100% [57], though such a high predictive value has not been achieved in all studies on the subject.

T cells

T cells are produced in the bone marrow by hematopoietic stem cells [58]. They migrate to the thymus where they undergo a selection process in which most of them die. They are thus called thymus-dependent lymphocytes, or T cells. During their development, T cells re-arrange genes encoding their antigen receptors, called the T cell receptor (TCR). The selection process ensures that the T cells that survive only bind major histocompatibility complex molecules, or HLA in humans, from the same organism, and that T cells that are capable of responding to self antigens are not produced, ensuring self-tolerance. HLA-molecules come in two forms, class I is expressed on all nucleated cells whereas class II is expressed on antigen presenting cells (APC) of the immune system. T cells with a TCR recognizing HLA class I will mostly develop into $CD8^+$ or cytotoxic T cells, while those that bind to HLA class II molecules will develop into $CD4^+$ or T helper cells. HLA class I molecules present peptides

from proteins produced in each cell, and will present viral peptides to $CD8^+$ T cells when a cell is infected. HLA class II molecules expressed on APC are loaded with peptides from proteins that the APC has ingested, for example by phagocytosis of bacteria, and these will be presented to $CD4^+$ T cells.

Helper T cells and cytotoxic T cells differ in their function. Cytotoxic T cells directly kill cells presenting foreign antigen on HLA class I once they have been primed by APCs, whereas helper T cells secrete cytokines that control other cells of the immune system and activate B cells and macrophages after they have encountered foreign antigen presented by an APC.

T cells that encounter their specific antigen:HLA complex will become activated through the TCR. A second signal is however required for the T cell to survive and become an effector T cell (Teff). In the absence of this second signal, the T cell will become anergic, that is unresponsive to further antigen stimulation, or undergo activation-induced cell death. The second signal is provided when a co-stimulatory receptor on the T cell called CD28 binds B7 molecules on the APC. The co-stimulatory signal is generally only provided when inflammation is present. When both signals are provided, T cells will upregulate a high-affinity IL-2 receptor and produce the cytokine IL-2 to drive its own proliferation. Upon activation, a protein called CTLA-4 is expressed, which is very similar to CD28 but instead delivers an inhibitory signal. It binds B7 molecules much more strongly than CD28 and makes the T cells less sensitive to further stimulation, which is essential in limiting the response of activated T cells (Fig 3).

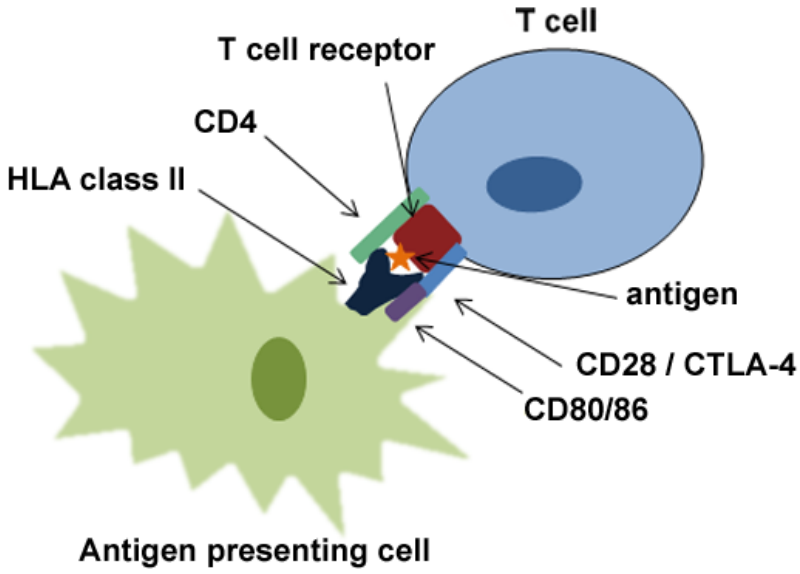

Figure 3. *Schematic illustration of antigen presentation to CD4 T cells. Activation of T cells requires antigen presentation through the T cell receptor when it binds its cognate antigen on an HLA class II molecule on an APC. In addition, co-stimulation is required by binding of CD28 expressed by the T cell to CD80/86 on the APC. If CTLA-4 is expressed on the T cell, it out-competes CD28 for CD80/86 binding and inhibits T cell activation.*

T cell subsets

A third signal also comes into play, mainly directing the differentiation of the T cell into a functional subset. This signal consists of cytokines in the local environment, usually produced by the cell presenting the antigen. The most well characterized subsets are called T helper (Th) 1 and Th2 [59], and there is further evidence of proinflammatory Th17 cells, adaptive Treg, as well as Th3 and Tr1 regulatory subsets. Th1 differentiation is driven by interferon (IFN)-γ and IL-12, and Th1 cells will be effective at inducing macrophage activation, opsonizing antibodies and generally a more cell-mediated immunity. Differentiation of Th2 cells is instead driven by IL-4, and the immune response to a Th2 polarized antigen will be humoral, that is mainly mediated through antibody actions. The effector cytokines of Th1 and Th2 cells inhibit the other type of T helper cell, while propagating a positive feedback loop favoring either Th1 or Th2 immunity once either has been established. Adaptive Treg are induced by transforming growth factor (TGF)-β signaling, and resemble naturally occurring Treg which will be described in detail in the next chapter [60-61], while Tr1 and Th3 cells are

induced by IL-10 signaling [62-63]. Th17 cells are generated in response to TGF-β signaling in an inflammatory milieu [64], and are important in immunity against extracellular bacteria and fungi [65]. This model of T cell lineages may be overly simplistic, however. It is possible that T cell heterogeneity is very complex, and that a single secreted cytokine or transcription factor is not sufficient to delineate lineages.

Th1 cells are thought to predominate in T1D [66]. Administration of cytokines that promote Th1 polarization has been shown to exacerbate T1D in animal models, while antibodies against these cytokines suppressed disease. Th1 cells also efficiently transfer T1D between animals. In contrast, a Th2 type response seems to be protective. Autoreactive T cells specific for islet antigens have been shown to be polarized toward a Th1 phenotype in patients with T1D, while islet-specific cells in healthy individuals are biased toward a Treg phenotype, secreting anti-inflammatory cytokines [67]. Furthermore, one of the hallmark cytokines associated with Th1 cells, IFN-γ, is crucial for β-cell destruction in T1D [68]. There is also a role for Th17-type immunity in T1D, since children with T1D have upregulated Th17 immunity in peripheral blood, with higher IL-17 secretion *in vitro* and higher IL-17 mRNA [69]. In addition, IL-17 potentiated both inflammatory and proapoptotic responses in human islets *in vitro*.

T cells are divided into naïve and memory T cells. Memory T cells are T cells that have encountered antigen in conjunction with co-stimulation and appropriate cytokine signaling from APC, and do not require co-stimulation to become activated when they encounter their antigen. Naïve and memory T cells are discriminated by expression of different isoforms of a surface receptor called CD45, where naïve cells express CD45RA and memory cells express CD45RO.

Regulatory T cells

The idea of a negatively regulating subset of T cells emerged in the '70s and established the field of suppressor T cells. However, suppressor T cells fell into disrepute with the arrival of new techniques and a spate of negative findings in the early '80s [70]. As suppressor T cells fell from grace, experiments in mice revealed that neonatal thymectomy led to wide-spread organ-specific autoimmunity, which could be prevented by transfer of spleen cells or thymocytes from healthy adult animals [71-72]. Soon after, it was shown that removal of T

cell subsets caused autoimmunity in mice and rats, and that the development of autoimmunity in these animals could be inhibited by other T cell subsets [73-74].

A great breakthrough was made when Sakaguchi and colleagues defined $CD4^+CD25^+$ T cells as natural Treg in mice, showing that transfer of spleen and lymph node cell suspensions devoid of $CD4^+$ T cells expressing the high-affinity IL-2 receptor CD25 caused multiple autoimmune diseases when inoculated into athymic mice but that co-transfer of $CD25^+$ cells prevented disease [75]. Identifying the human $CD4^+CD25^+$ Treg counterpart proved more problematic, since activated T cells express CD25 in humans, but it was eventually identified as $CD4^+CD25^{hi}$ T cells [76]. IL-2 has been shown to be essential for Treg homeostasis in mice [77].

Treg have been shown to suppress activation, proliferation and cytokine secretion of $CD4^+$ and $CD8^+$ T cells even in the absence of APC *in vitro* [78-80]. They also suppress proliferation in B cells, as well as production of antibodies and class switching [81-82]. Furthermore they inhibit natural killer and natural killer T cell cytotoxicity [83-84], as well as the maturation and function of dendritic cells (DC) [85]. Treg thus regulate a wide array of immune responses, and it comes as no surprise that they are central in protection against autoimmune diseases, allergy and in maintaining tolerance to the fetus during pregnancy [86]. Treg frequencies have been shown to be diminished in active systemic lupus erythematosus [87], and decreased Treg numbers have been correlated with disease severity in several cases. In multiple sclerosis, both increased and decreased frequencies of forkhead box P3 $(FOXP3)^+$ cells have been observed [88-89], while patients with rheumatoid arthritis seem to have increased numbers of $FOXP3^+$ Treg [90]. Several studies have also failed to find any evidence of altered frequencies of Treg in multiple sclerosis, rheumatoid arthritis and T1D [91-94]. Moreover, the suppressive function of Treg has been found to be diminished in patients with multiple sclerosis, psoriasis, myasthenia gravis and autoimmune polyglandular syndrome type 2 [95-98]. A subset of Treg expressing CD39 is considered highly suppressive, and this subset was decreased in patients with multiple sclerosis [99-100].

Phenotype of Regulatory T cells

CTLA-4 was found to be expressed on human $CD4^+CD25^+$ cells in one of the first reports on human Treg [101], after it was shown to be constitutively expressed on murine Treg [102-103]. As CTLA-4 deficiency impairs Treg suppressive function *in vivo* and *in vitro* in mice

[104], it is thought to be important for Treg suppressive function, apart from being used as a marker of Treg in flow cytometry. CD39 is another surface protein thought to be involved in Treg-mediated suppression and has been associated with more suppressive subsets of Treg [99,105].

The transcription factor FOXP3 has been shown to control Treg development and is highly expressed in $CD4^{+}CD25^{hi}$ cells in both rodents and humans [106-107]. Specifically, FOXP3 induces expression of CD25, CTLA-4, glucocorticoid-induced TNFR-related protein (GITR) and CD39 while repressing production of IL-2, IFN-γ, tumor necrosis factor (TNF) and IL-4 [108-109]. However, FOXP3 is expressed transiently after activation of conventional T cells without conferring regulatory function [110-112]. Activated conventional T cells transiently expressing FOXP3 can be distinguished from committed suppressive Treg by analyzing demethylation of the FOXP3 locus [113-114], since naturally occurring Treg have a demethylated FOXP3 promoter region whereas activated T cells express FOXP3 despite maintaining partial methylation of the gene [115]. $FOXP3^{+}$ Treg with stable suppressive activity can reportedly be generated using a DNA methyltransferase inhibitor [116]. It was recently shown that commitment to a stable $FOXP3^{+}$ Treg lineage is initiated during early stages of thymic development through this epigenetic mechanism [117]. Miyara *et al* used CD45RA and FOXP3 expression to delineate functionally distinct subsets of Treg, demonstrating that $FOXP3^{hi}CD45RA^{-}$ cells are highly suppressive while $FOXP3^{lo}CD45RA^{-}$ cells are not suppressive [118]. The activated $CD45RA^{-}FOXP3^{hi}$ population was later demonstrated to be defined by CD147 expression [119].

Expression of the IL-7 receptor CD127 has been shown to inversely correlate with FOXP3 expression on Treg [120], and expression of CD127 in conjunction with CD25 is used to discriminate Treg from activated T cells [121]. It was recently shown that IL-7 treatment of $CD25^{+}CD127^{+}$ cells produced cells with the classic $CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+}$ phenotype, and this may be important for the survival of adaptive Treg in the periphery [122].

HLA-DR is expressed on T lymphocytes after activation. Treg expressing HLA-DR have been shown to inhibit T cell proliferation and cytokine production in an early contact-dependent manner, while $HLA\text{-}DR^{-}$ instead secreted IL-10 and IL-4 and induced a late suppression of proliferation [123]. $HLA\text{-}DR^{hi}CD45RA^{-}$ Treg in particular have been found to be more suppressive than DR^{lo} or DR^{-} subsets [124].

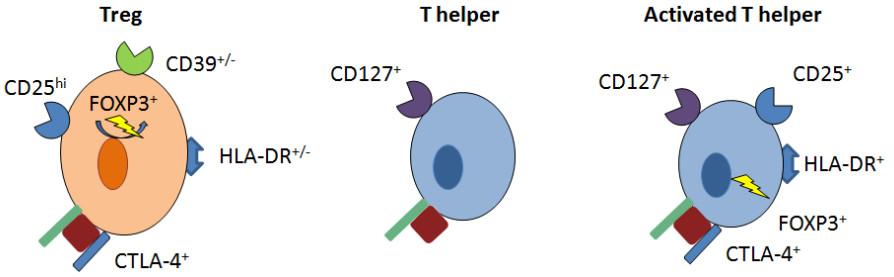

Figure 4. *Illustration of key differences in expression of surface markers and the transcription factor FOXP3 between Treg and T helper cells. Treg express high levels of CD25, FOXP3 and CTLA-4, and may express CD39 and HLA-DR, but not CD127. Conventional T cells do not express high levels of CD25 but express CD127. Activated conventional T cells additionally express common markers of Treg like CD25, FOXP3, CTLA-4 and HLA-DR.*

Treg are also known to express CD69 and CD62L. Subsets of effector Treg express ICOS, which differentiates between IL-10 and TGF-β producing Treg. LAG3 and CD95 are also expressed by Treg and are involved in suppressive function through inhibitory signals through HLA class II molecules and induction of apoptosis in conventional T cells, respectively [125].

Adaptive Regulatory T cells

While the classical, naturally occurring Treg mature in the thymus and commit early on to a regulatory program, there is some evidence of Treg generation from $CD4^+CD25^-$ cells in the periphery [126-127]. These adaptive Treg are thought to be induced under various circumstances such as cytokine or low-dose antigen stimulation [61,128]. Some of the peripherally generated $FOXP3^+$ regulatory cells are potentially transient or less stable than natural Treg. Adaptive Treg are similar to naturally occurring Treg phenotypically and functionally in that they express high levels of CD25 and CTLA-4 and require cell contact to exert suppression. However, it has been reported that TCR stimulation alone does not induce FOXP3 expression, while the presence of TGF-β during TCR stimulation does induce FOXP3 expression but does not confer suppressive function [112]. Expression of Helios was proposed to differentiate natural, thymically derived Treg from Treg induced in the periphery [129]. However, further studies have provided evidence that Helios is expressed on peripherally

induced Treg as well, and that both Helios$^+$ and Helios$^-$ thymically derived Treg coexist in the periphery [130-131].

Two distinct subsets of Treg exist, which may be classified as adaptive. One subset is the Th3 cell, which is induced in the oral mucosa in response to low-dose antigen and reportedly secretes TGF-β [63]. The other subset is termed type 1 regulatory T cells, or Tr1, and is induced by antigen stimulation in an IL-10 dependent process *in vitro* and *in vivo* and secrete both IL-10 and TGF-β [132]. Neither cell type is very well defined, and their relationship with CD4$^+$CD25$^+$ Treg is unknown, nor is it known whether these cells originate from similar precursors. The main mechanism of action of Tr1 cells seems to be their secretion of IL-10, but they have also been shown to lyse APC through a granzyme B- and perforin-dependent pathway [133]. CD49b and LAG-3 were very recently established as markers of Tr1 cells, which might finally facilitate further study of this subset [134].

Natural Treg have been shown to be able to confer suppressive capability in conventional CD4$^+$ T cells through a cell contact dependent mechanism, a phenomenon called infectious tolerance. The suppression exerted by the induced adaptive Treg is partially mediated by TGF-β and is not cell contact dependent [135]. Another report demonstrated that conventional T cells made suppressive through infectious tolerance produced IL-10 and were again contact-independent, similar to Tr1 cells discussed above [136].

Mechanisms of Treg-mediated suppression

Treg require antigen-specific signaling through their TCR to initiate suppression, but once activated their suppression is not antigen-specific, which means that they can suppress responder T cells of any antigen-specificity [137]. Both regulatory cytokines and cell contact-dependent mechanisms have been implied in the mechanism of action of Treg. CD25$^+$ Treg were shown to control responder T cells through the secretion of IL-10 in mice [138]. This would be a plausible mechanism of action since IL-10 has previously been shown to induce long-lasting anergy in CD4$^+$ T cells [139]. However, some findings indicate that suppression is cytokine independent but cell contact dependent [137]. Cytokines may be involved in Treg mechanism of action despite the apparent cell-contact dependent means of suppression, since for example TGF-β is expressed on the cell surface of Treg rather than being secreted when Treg are stimulated with antigen via APC [82].

Treg also mediate suppression through inhibition of IL-2 at the mRNA level in responder T cells [80]. It has further been suggested that Treg might outcompete conventional T cells for binding of IL-2 through their higher expression of the high-affinity IL-2 receptor and thus inhibit their proliferation, but several findings argue against this proposed mechanism [140].

CTLA-4 is a CD28 homologue that unlike CD28 delivers a negative costimulatory signal which suppresses T cell activation [141-142]. Its expression is induced on conventional T cells by activation [143], and its ligation blocks IL-2 production, IL-2 receptor expression and cell cycle progression of activated T cells [144]. One of the proposed mechanisms of actions of CTLA-4 on Treg is its ability to induce indolamine 2,3-dioxygenase on DC [145]. Indolamine 2,3-dioxygenase converts tryptophan to kynurine, which has potent local immunosuppressive effects [146], while tryptophan deprivation in itself prevents cell division in activated T cells. Furthermore, Treg are capable of down regulating CD80/86 expression on DC and their ability to do so is impaired by CTLA-4 deficiency [104]. In contrast, CTLA-$4^{-/-}$ Treg have been shown to retain suppressive function through a TGF-β dependent pathway, even though wildtype Treg are not dependent on TGF-β for suppressive function [147].

CD39 expressed on Treg acts to remove a proinflammatory signal while simultaneously generating an immunosuppressive stimulus in conjunction with CD73. They jointly catalyze the generation of cyclic adenosine monophosphate (cAMP) and eventually adenosine, which has immunosuppressive effects, from extracellular adenosine triphosphate (ATP) [105,148]. Additionally, CD39-expressing Treg suppress ATP-driven maturation of DC which is another potential pathway of immune regulation [99]. Extracellular ATP is released by damaged cells as an indicator of trauma or cell death, and is a mediator of proinflammatory signals [149]. Monocytes release IL-1β in response to ATP signaling through purinergic P2 receptors [150-151], which activates the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome and consequently caspase-1 [152]. In contrast to mice, only a subset of human Treg expresses CD39. IL-1β has been shown to be crucial for induction of proinflammatory Th17 cells [153-154]. Th17 cells have in turn been known to be resistant to Treg-mediated regulation, but it was recently discovered that only the $CD39^{+}$ subset of Treg is able to suppress IL-17 production [100]. Decreased levels of CD39-expressing Treg have been observed in patients with multiple sclerosis [99].

Treg also harbor high concentrations of cAMP intracellularly. cAMP is a mediator of cell-contact dependent suppression via the formation of gap junctions between Treg and responder

T cells as demonstrated in experiments where suppression was abrogated by either a cAMP antagonist or gap junction inhibitors [155]. Treg can also control Teff directly by inducing apoptosis through release of granzyme A and perforin [156]. Treg were also able to induce apoptosis of Teff through cytokine deprivation in a mouse model [157], and this pathway required the proapoptotic protein Bim. Forced expression of Bcl-2, which counteracts the effects of Bim, rendered Teff resistant to this form of regulation. Whether this is true for humans is yet to be determined.

Finally, Treg have been shown to form stable, long-lasting clusters around DC. The Treg-DC interaction was significantly longer lasting than Teff-DC interactions in humans *in vitro* [158]. Studies in mice have shown that Treg form clusters with DC that prevent Teff-DC interaction and priming of Teff [159-160]. These findings suggest that Treg are able to sequester DC presenting self antigen from potentially pathogenic Teff, and consequently prevent their activation.

Regulatory T cells in T1D

The role of Treg in T1D has been studied extensively over the last decade, and like in several autoimmune diseases, both the frequency and function of Treg has been found to be affected in different settings of T1D. There are however contrasting findings that may in part be due to differences in experimental design and study populations. Kukreja *et al* demonstrated deficiencies in the frequency of $CD4^+CD25^+$ T cells in patients with both recent-onset and long-standing T1D [161]. Lindley *et al* found that while the frequencies of $CD4^+CD25^+$ Treg in peripheral blood from patients with recent onset T1D were normal, their suppressive function was impaired, and T1D Treg expressed higher levels of intracellular CTLA-4 [93]. Brusko *et al* produced similar observations, and additionally found no difference in $CD4^+CD25^{hi}$ Treg frequency in patients with different disease duration [162]. When monoclonal antibodies against FOXP3 became commercially available, the same group demonstrated that patients with T1D and subjects at risk of developing T1D had similar frequencies of $FOXP3^+$ Treg as healthy controls [94]. Defining Treg as $CD25^{hi}CD127^{lo}$ is more robust than the definition using only $CD25^{hi}$, and frequency of $CD25^{hi}CD127^{lo}$ Treg was also similar in patients with T1D and controls [94,163]. Another study of Treg in T1D found neither *in vitro* functional defects nor changes in frequency of $CD4^+CD25^{hi}$ Treg in T1D subjects [164]. The contrasting findings could be due to different approaches to discriminate

Treg, for example both the Kukreja and Lindley reports defined Treg as $CD4^+CD25^+$ while the studies by Brusko and Putnam used the $CD4^+CD25^{hi}$ definition demonstrated to better define Treg in humans. Furthermore the age-matching of patients and controls in the different studies varied considerably. Treg from patients with T1D might also be more prone to apoptosis [165], and instability of the Treg pool could potentially contribute to autoimmunity if antigen specific Treg are not maintained. The current consensus is that the frequency of Treg in T1D is not affected, but rather their function seems to be impaired.

Analysis of distinct Treg subsets based on CD45RA and FOXP3 expression as described by Miyara *et al* revealed that recent-onset T1D children had higher frequencies of non-suppressive $CD45RA^-FOXP3^{lo}$ cells [166]. The non-suppressive cells were additionally shown to produce IL-17 in these subjects. However, this was not the case in adults with T1D, where neither CD45RA/FOXP3 subsets nor FOXP3 gene demethylation in Treg was different from controls [167]. It has also been demonstrated that patients with T1D generate both polyclonal and islet antigen-specific adaptive Treg normally [168-169].

Treg on the front lines

It was shown in a transgenic mouse model that the frequency of Treg in the pancreas dropped dramatically preceding onset of T1D [170]. The frequency of Treg in the pancreas is hard to define in humans, because acquiring pancreatic biopsies is very difficult. Immunohistochemical analysis of pancreatic biopsies taken post mortem from patients with T1D revealed infiltration by mainly $CD8^+$ cells, but also $CD4^+$ cells, and crucially FOXP3 expression was detected very rarely in only a single patient [171], which indicates a potential absence of Treg in the pancreas in T1D. Phenotypical and functional characterization of Treg from pancreas-draining lymph nodes of patients with T1D demonstrated functional and numerical defects in $CD25^{hi}CD27^{lo}FOXP3^+$ Treg from pancreatic lymph nodes but not from peripheral blood [172]. However, the frequency of cells with the Treg-specific FOXP3 demethylation was similar both in peripheral blood and pancreatic lymph nodes of patients with T1D and controls. In addition, proinsulin-specific Treg-mediated regulation was impaired in pancreatic lymph nodes of patients with T1D as demonstrated in suppression assays where Teff were stimulated by DC exposed to proinsulin. It is thus possible that there are local deficiencies of Treg in the pancreas of patients with T1D, and one should keep in

mind that observations made in peripheral blood might not represent the situation in the target organ.

Resistant Teff in T1D

Effector T cells of patients with T1D have been shown to be resistant to Treg-mediated regulation [163,169]. This finding has important implications for any clinical applications of *ex vivo* expanded Treg in the treatment of T1D. It also makes the relevance of analyzing frequencies of Treg in the T1D setting questionable, but also motivates further research into Treg deficiency in T1D. Multiple factors can contribute to Teff resistance to Treg-mediated suppression, such as hyperactivation of the phosphatidylinositol 3-kinase – Akt pathway in Teff, Toll-like receptor signaling, cytokines including IL-2, IL-7 and IL-21, and GITR signaling [173-174]. APC can influence resistance to Treg-mediated suppression through alteration of differentiation and proinflammatory cytokine production, but since the experiments on which these observations are based were performed using anti-CD3/CD28-coated beads, APC cannot explain resistance in this case [163,169]. Schneider *et al* further demonstrated that resistance to suppression was not due to higher numbers of $CD45RO^+$ memory T cells in Teff from patients with T1D, nor was it affected by the HLA class II type of the subjects [169]. Furthermore, as resistance to suppression was not transferred to control Teff co-cultured with T1D Teff, soluble factors are an unlikely mechanism of resistance to suppression.

Cytokines

Cytokines are signaling molecules that usually act in the local microenvironment of the cell secreting them. They are key players in differentiation of various T cell subsets, as well as in mediating their effector functions and in directing the immune response against various pathogens. As discussed previously, Th1 cells predominate in T1D and one of the main cytokines secreted by Th1 cells, IFN-γ, has been associated with the insulitic lesion in T1D [175]. Cells from peripheral blood of patients with T1D also produce mainly Th1 associated cytokines such as IFN-γ and TNF, and low levels of Th2 associated cytokines IL-4 and IL-10 [176]. At clinical onset of T1D, patients have higher serum levels of IL-1β, IL-1α, TNF and IL-6 compared to healthy controls [177]. It has also been shown that IL-1, TNF and IFN-γ are cytotoxic to β-cells *in vitro* [178].

IL-1β induces dysfunction and apoptosis in β-cells, and inhibits release of insulin [179-180]. There is also indication that IL-1β is expressed by pancreatic islets under hyperglycemic conditions, and that IL-1β has a role in mediating deleterious effects of high glucose concentrations on β-cells [181]. The active form of IL-1β is produced from pro-IL-1β through cleavage by caspase-1, which is activated by a protein complex called the NALP3 inflammasome [182]. Macrophages of patients with T1D have been shown to spontaneously secrete IL-1β and IL-6, which in turn induces differentiation of pathogenic IL-17 secreting cells, suggesting that IL-1β may be a link between innate and adaptive immunity in T1D [183]. IL-1β has also been shown to drive proliferation and cytokine production of Teff and memory T cells, attenuate Treg suppressive function and allows autoreactive Teff to escape suppression in mice models [184]. These strong indications of IL-1β involvement in T1D pathogenesis lead researchers to trial an IL-1 receptor antagonist in patients with T1D, and were the rationale for our hypothesis that NALP3 inflammasome mutations could affect T1D risk and severity as examined in paper IV.

NALP3 Inflammasomes

The term inflammasome was coined in 2002 by a group that discovered a protein complex consisting of ASC adaptor, NALP1, caspase-1 and caspase-5, which processed pro-IL-1β [185]. Although the diversity and biochemistry of inflammasomes is not fully understood, they are divided into NALP1, NALP3 and IPAF inflammasomes [186]. The NALP3 inflammasome consists of NALP3, ASC adaptor and caspase-1, and has high pro-IL-1β-processing activity (Fig 5) [182]. Cardinal/TUCAN/ Caspase recruitment domain-containing protein 8 (CARD8) has also been suggested as a binding partner of the inflammasome but its role is still debated. NALP3 is involved in sensing danger signals, signs of damaged or stressed tissues that are thought to be involved in how the immune system mounts a response to pathogens but not to, for example, commensal bacteria.

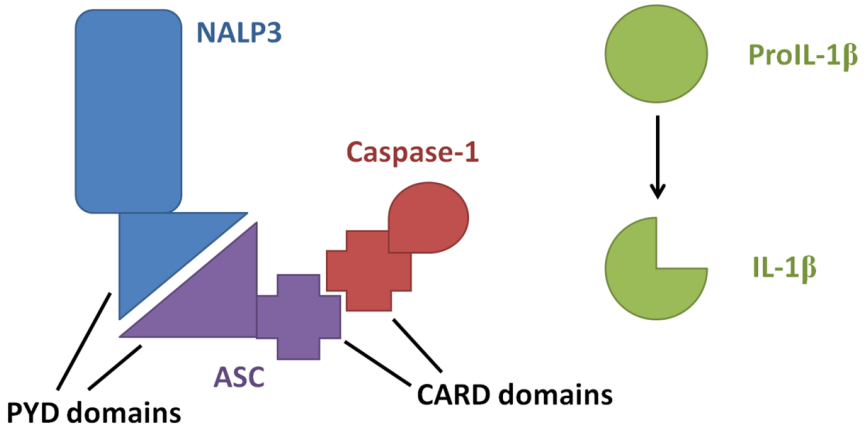

Figure 5. *Schematic illustration of the NALP3 inflammasome protein complex assembly, which results in proteolytic processing of proIL-1β to form active IL-1β. A PYD domain of the adaptor protein ASC binds another PYD domain of NALP3 and a CARD domain of ASC binds another CARD domain of Caspase-1 to form the assembly.*

Inflammasome activation

The inflammasome is formed spontaneously if cellular integrity is compromised [186]. This can be prevented by artificially mimicking potassium levels found in the cytosol of healthy cells, which indicates that subphysiological levels of potassium is required for spontaneous inflammasome formation. This is supported by the fact that other activators of the inflammasome induce K^+ efflux.

As mentioned previously, ATP is released by damaged cells as a sign of cellular injury and is a potent proinflammatory mediator. Extracellular ATP binds the P2X7 receptor and thereby activates the NALP3 inflammasome through decreased intracellular K^+ levels [152]. Interestingly, rat β-cells can release ATP from secretory granules through kiss-and-run exocytosis [187].

Uric acid is contained in the cytosol of cells at very high concentrations and is released by injured or dying cells [186]. When released, it is thought to form monosodium urate crystals, which act as an adjuvant. The biological activity of monosodium urate crystals is dependent on the NALP3 inflammasome, which is stimulated by the crystals to produce active IL-1β [188]. Removal of uric acid has been shown to reduce activation of cytotoxic T cells and the proliferation of autoreactive T cells in a transgenic diabetes model [189]. Uric acid relase also

occurs in DC in the presence of the adjuvant alum [190], which has also been shown to be a direct activator of the inflammasome [191].

Immunization with alum as an adjuvant leads to increased antigen-induced T cell proliferation resulting from increased production of IL-1, and anti-IL-1 antibodies can inhibit antigen-specific T cell responses after immunization with alum as an adjuvant, but not when Freund's complete adjuvant is used [192-193]. Alum has been shown to activate caspase-1 and produce active IL-1β *in vitro*, and this is NALP3 inflammasome dependent [191,194].

Link to T1D and GAD-alum treatment

Mutations of the NALP3-encoding NOD-like receptor family, pyrin domain containing 3 (NLRP3) gene or the CARD domain may result in spontaneous processing and secretion of IL-1β [195]. For instance, the single nucleotide polymorphism (SNP) Q705K in the NALP3 gene is a gain of function mutation with moderate effect, leading to excessive IL-1β production and inflammasome hyperactivity [196]. While IL-1β is involved in induction of Th17 cells, inflammasome-mediated alum adjuvanticity favors a Th2 response. The mechanism behind this effect is unclear.

There is a distinct role for the inflammasome and IL-1β in promoting and directing adaptive immunity. Despite this, most diseases with established inflammasome hyperactivity lead to autoinflammatory syndromes that rely completely on innate immunity, with no involvement of adaptive immunity. Considering its effect on T helper subsets and direct cytotoxic effects of its end product IL-1β, there is a strong rationale for inflammasome involvement in the pathogenesis of T1D. Furthermore, since alum's effects are known to be inflammasome-mediated, and because alum is used as an adjuvant in GAD-alum treatment, modifications of inflammasome signaling might affect the efficacy of GAD-alum treatment.

Immune intervention in T1D

Shortly after T1D diagnosis, the pancreas is still able to produce significant amounts of insulin, sometimes even many years after diagnosis [197]. During this time, immune intervention can potentially preserve residual β-cell function, reducing the patient's reliance on exogenous insulin and improving metabolic control, which in turn limits the risk of both acute and secondary complications. Thus, intervention may be beneficial even if it does not

completely stop the autoimmune process. Early attempts at inteverntion included trials of corticosteroids, immunosuppressants and plasmapheresis [198-200], while recent attempts are targeting specific aspects of immune signaling, including cytokines and effector molecules expressed by lymphocytes, through the use of monoclonal antibodies or receptor antagonists, or modulation of immune responses to T1D-related autoantigens.

Anti-CD3

CD3 is a protein complex expressed on all T cells and is involved in T cell activation. It has been demonstrated that administration of anti-CD3 monoclonal antibodies induces T cell depletion and significantly inhibits the autoimmune process in non-obese diabetic (NOD) mice [201]. It was recently demonstrated in a mouse model that anti-CD3 treatment selectively targets conventional T cells and not $FOXP3^+$ Treg [202]. Early clinical trials of anti-CD3 in a small number of patients with T1D showed promising results such as preservation of insulin secretion and lower insulin requirement during the single year the patients were followed [203]. The treatment was accompanied by several side-effects, including cytokine release, anemia, moderate fever and rashes, but no long-term effects. The clinical effect was later shown to persist for 2 years [204], but an increased dose was associated with greater adverse events with no improvement in clinical efficacy [205]. Two subsequent phase III trials performed using Teplizumab and Otelixizumab failed to reach primary outcomes of decreased HbA1c levels and insulin dose, or differences in C-peptide levels, respectively [206-207]. However, a reduced loss of C-peptide was demonstrated at the 2-year follow-up of one of these phase III trials (Protégé trial) [208]. Currently, the international consortium TrialNet is recruiting non-diabetic individuals at risk of developing T1D to participate in a prevention trial using Teplizumab, and further immune intervention trials at onset are being discussed.

Anti-CD20

Even though T1D is mediated by T cells, B cells have been implicated in the pathogenesis of the disease since they are present in pancreata of patients with T1D and are likely to act as APC. B cell depletion using Rituximab, an anti-CD20 monoclonal antibody, was tested in patients with recent onset T1D and showed some improvement of C-peptide secretion 3 months after treatment, while the rate of C-peptide loss was similar in placebo treated

individuals after 6 months [209]. Treated patients also had lower levels of glycated hemoglobin and required less insulin, but worryingly, the frequencies of $CD19^+$ B cells did not recover to baseline numbers 1 year after treatment. There is therefore a risk of chronic immune suppression as a consequence of anti-CD20 treatment.

CTLA4-Ig

As previously discussed, T cells require two signals to become fully activated; TCR ligation as well as binding of CD28 to B7 molecules on APC. CTLA-4 Ig or Abatacept is a CTLA-4-immunoglobulin fusion protein that selectively binds B7 molecules on APC and prevents interaction with CD28, thereby interfering with early T cell activation, proliferation and survival. It targets mainly naïve T cells since they are more reliant on costimulation than effector memory T cells, which are presumably less affected by the treatment. A clinical trial of Abatacept in recent onset T1D patients showed a significant effect on C-peptide secretion in treated individuals, but the rate of decline in C-peptide levels was the same in placebo treated individuals a few months after treatment and throughout the treatment period despite continuous infusions with Abatacept over a 2 year period, suggesting that loss of insulin secretion is delayed but eventually proceeds at a similar rate in treated patients [210].

IL-1 blockade

An IL-1 receptor antagonist, Anakinra, has been used in clinical trials in Type 2 Diabetes, with great promise [211]. Treated patients showed improved glycemic control, reduced inflammation, increased insulin sensitivity and there were also signs of beneficial effects on β-cells, with a higher ratio of insulin/proinsulin in treated patients. In T1D, β-cells might produce Il-1β under hyperglycemic conditions, which could perpetuate inflammation in the pancreas. IL-1β has additional deleterious effects on β-cells, including induction of secretory dysfunction and apoptosis. It is also thought to act as a link between innate and adaptive immunity, specifically promoting differentiation of Th1 and Th17 cells and allowing Teff to proliferate in the presence of Treg. Clinical trials of Canakinumab, an anti-IL-1 monoclonal antibody, and Anakinra were performed in patients with recent onset T1D [212]. Neither drug had any significant effect on C-peptide secretion, and while Canakinumab was well tolerated, Anakinra treated individuals had numerous injection site reactions due to daily injections of

Anakinra. The authors argue that IL-1 blockade might be useful in combination with treatments targeting adaptive immunity.

IL-2 & Rapamycin

IL-2 is a survival and growth factor for T cells, and Treg expansion in particular is highly dependent on IL-2 stimulation. Rapamycin inhibits proliferation of mainly Th1 and Th17 cells, both of which have been implicated as effector cells in T1D pathogenesis. Combination treatment with Rapamycin and IL-2 was effective in the NOD mouse model of T1D, which prompted a phase I clinical trial of IL-2/Rapamycin in patients with T1D within 4 years of diagnosis. The treatment increased the frequency of Treg within one month, but this increase was transient and abated when IL-2 administration was withdrawn. In addition, C-peptide levels dropped transiently in all 10 treated patients despite the increase in Treg frequency [213]. The trial highlights the difficulty of translating findings in animal models to the clinic.

Anti-TNF

TNF is a Th1-associated cytokine known to potentiate direct cytotoxic effects of IL-1β and IFN-γ on β-cells, and results from animal models indicate that TNF over expression in β-cells worsens insulitis, and that this is abrogated in TNF receptor deficient mice [214]. Etanercept is a soluble recombinant fusion protein of the TNF receptor that binds TNF, removing it from the circulation, thereby preventing the biological activity of TNF. The use of Etanercept in a pilot study including recent-onset children and adolescents with T1D demonstrated improved insulin secretion after the 2 year treatment in the etanercept group, while it decreased in the placebo group over the same time period [215]. Insulin requirement was concomitantly decreased in treated patients and increased in the placebo group. More extensive clinical trials will be needed to confirm these promising initial findings.

Insulin

Studies in animal models indicated that oral administration of insulin could prevent onset of T1D. However, oral insulin administration in recent onset T1D had no effect on insulin secretion or insulin requirement [216]. Nevertheless, there was a suggestion of benefit on diabetes incidence in a subgroup with very high titers of antibodies against insulin [217]. It

might thus be possible that oral insulin can delay disease onset in subjects at high risk of developing T1D. Nasal administration of insulin was tested in a separate trial but could not prevent or delay onset of T1D [218].

DiaPep277

DiaPep277 is a peptide from heat shock protein 60 which is expressed in secretory granules of β-cells. T cells reactive to heat shock protein 60 have been shown to be of a Th2 phenotype, leading to the hypothesis that treatment with DiaPep277 could have immunomodulatory effects. After initial findings suggested a positive effect on preservation of insulin secretion, a phase II trial failed to demonstrate any effect of DiaPep277 on β-cell function or metabolic control [219-220]. Despite this, a global phase III trial in 457 patients with recent-onset T1D showed positive effects on preservation of insulin secretion and metabolic control after 2 years of treatment [221], but remarkably only after glucagon stimulation and not after a mixed meal tolerance test which is regarded as more relevant clinically.

Treg Immunotherapy

Autoimmunity can be prevented by transfer of Treg in mice models, which has created interest for similar treatment strategies in human autoimmune disease [222]. Studies in mice also demonstrated that a very small amount of transferred polyclonal Treg respond and proliferate in the pancreatic lymph nodes, highlighting the need for antigen specificity of Treg used for immunotherapy. The HLA class II DR and DQ genes that confer risk of T1D have been suggested to be deficient in antigen binding [223]. In addition, a T1D-associated variant of the preproinsulin gene effects thymic expression of insulin [224]. Taken together, this may influence thymic generation and TCR repertoire of Treg in patients with T1D. Engineering the TCR of autologous Treg might therefore be a potential strategy to enhance Treg cell therapy in the future. Treg expanded from umbilical cord blood have been shown to be safe and effective in treatment of graft-versus-host disease after umbilical cord blood transplantation [225]. The recent discovery of CD49b and LAG-3 as markers of Tr1 cells has facilitated their use in ongoing clinical trials [134].

Cellular therapy utilizing *ex vivo* expanded Treg to prevent autoimmunity as well as graft-versus-host disease due to bone marrow transplantation is under investigation [226-227]. A

phase I dose-escalating clinical trial of *ex vivo* expanded autologous polyclonal Treg in patients with recent onset T1D aged 18-35 years, using a very similar protocol to the one used in paper II in this thesis, is underway [228]. Specifically, $CD4^{+}CD25^{hi}CD127^{lo}$ cells are sorted using good manufacturing practice grade monoclonal antibodies, and expanded in the presence of anti-CD3/CD28 coated beads and IL-2 [229]. The study will provide important information regarding cell dose, safety and potential efficacy, though it remains to be seen whether the infused polyclonal Treg contains sufficient numbers of antigen specific Treg, or whether they will be able to induce sufficient bystander suppression to reverse or inhibit established organ specific autoimmunity.

Apart from direct transfer of Treg, treatment with drugs that affect Treg frequency have been used in clinical trials. Corticosteroid treatment of SLE was associated with increased numbers of Treg [230], while CD25-blocking antibody Daclizumab resulted in decreased Treg numbers in multiple sclerosis that was not related to clinical outcome [231]. In contrast, treatment of rheumatoid arthritis with Infliximab, which blocks the TNF receptor, induced increased frequencies of Treg that did correlate with improved clinical outcome [232]. Early studies of GAD-alum treatment, which is examined extensively in this thesis, showed an increased ratio of $CD25^{+}/CD25^{-}$ cells in latent autoimmune diabetes in adults (LADA) patients which correlated with increased fasting C-peptide levels [233]. However, the use of $CD25^{+}$ to delineate Treg in humans has severe limitations as discussed previously. Further studies of GAD-alum treatment in children with recent-onset T1D performed in our lab revealed increased frequencies of $CD25^{hi}FOXP3^{+}$ cells in treated patients upon antigen recall [234]. However, since cells were exposed to antigen for 18 hours prior to acquisition, it cannot be excluded that increased expression of FOXP3 was due to activation of conventional T cells.

GAD_{65} as an immunomodulator in T1D

Several attempts at using GAD_{65} to modulate the immune response in T1D by inducing T cell tolerance to GAD_{65} have been made in animals and humans since it was discovered that GAD_{65} is a major autoantigen in T1D.

Results from animal models

Early studies revealed that intraperitoneal injection of GAD_{65} in neonatal NOD mice significantly delayed the onset of T1D [235]. Nasal administration of GAD_{65} to NOD mice induced a marked shift to a Th2 phenotype in antigen specific T cells while decreasing insulitis and long-term diabetes incidence [236]. It was also demonstrated that treatment with GAD_{65} peptides induced antigen specific Th2 responses which spread to T cells specific for other antigens in an infectious manner [237]. This was accompanied by a decrease in long-term disease incidence. Finally, it was shown that intravenous administration of GAD_{65} suppressed an ongoing diabetogenic response in NOD mice, and that this was mediated by antigen specific Treg with a Th2 phenotype [238]. This conclusion was based on experiments where $CD4^+$ T cells from mice treated with GAD_{65} were transferred with diabetogenic spleen cells into irradiated NOD mice, where transfer of T cells from GAD_{65} treated mice prevented diabetes. However, since the T1D-suppressing cells were not further characterized using i.e. CD25 to determine whether they were indeed Treg, it is uncertain whether the prevention of T1D was truly mediated by Treg or if the previously demonstrated Th2 shift could be responsible for disease prevention.

Clinical trials of GAD_{65}

Phase I trial

Following studies in mice, unformulated recombinant human GAD_{65} was tested in 16 healthy males who received single subcutaneous injections of GAD_{65} or placebo to assess safety and tolerability [239]. The study tested increasing doses of GAD_{65}, from 20µg to a maximum of 500µg, and no significant treatment-related adverse events were identified at any dose-level nor did the treatment induce any autoantibodies to GAD_{65}, insulin or IA-2.

Early phase II trials

Recombinant human GAD_{65} was formulated in alum, commonly used in vaccines as an adjuvant (GAD-alum), for clinical use in humans. Aluminium hydroxide induces humoral Th2 responses when used as an adjuvant, rather than cell-mediated Th1 immunity [240], which should be beneficial in a Th1-associated setting like T1D. The mechanisms through which aluminium adjuvants exert their effect and induce Th2-type immunity have been

unclear. It appears the adjuvanticity is achieved through depot formation, increased targeting of the antigen to APC, stimulation of immune competent cells through activation of complement, as well as activation of macrophages [241]. More recently it has been demonstrated that the NALP3 inflammasome is crucial for the immunostimulatory properties of aluminium adjuvants, and that the inflammasome pathway can induce adaptive humoral Th2 responses [191].

LADA phase II trial

The first phase II trial of GAD-alum was a dose-finding study in LADA patients [233]. Forty-seven patients were randomized to receive placebo or 4, 20, 100 or 500µg of GAD-alum in subcutaneous injections. Safety evaluation including neurology, β-cell function, diabetes status, hematology, biochemistry and cellular and humoral immunological markers was carried out over 24 weeks. The results of the study showed that patients receiving 20µg of GAD-alum at 1 and 4 weeks had higher fasting C-peptide levels at 24 weeks compared to placebo, as well as higher fasting and stimulated C-peptide compared to baseline levels. The ratio of $CD4^+CD25^+/CD4^+CD25^-$ cells was also increased in the 20µg group. None of the patients had serious treatment-related adverse events, and the study established GAD-alum treatment as safe and effective in humans.

Phase II trial in children

The first clinical trial of GAD-alum in children with T1D was a randomized, placebo-controlled, double-blinded phase II trial which included 70 patients with T1D aged 10-18 years with less than 18 months disease duration [242]. Inclusion criteria were fasting C-peptide levels above 0.1nmol/l and detectable GAD autoantibodies at inclusion. Patients were randomized to receive subcutaneous injections of either GAD-alum (n=35) or placebo (alum only; n=35) at day 0 and 4 weeks later. A significant effect on preservation of both fasting and stimulated C-peptide levels was observed 30 months after treatment. The effect was most pronounced in a subgroup that had a disease duration of less than 6 months. A four-year follow-up of the study revealed a persistent effect on fasting C-peptide levels in patients with shorter disease duration at inclusion, and no serious treatment-related adverse events were reported [243].

Our group performed immunological studies of the patients included in the phase II trial in order to identify potential biomarkers of efficacy as well as to elucidate the immunological mechanism of GAD-alum treatment of T1D. GAD-alum treatment was demonstrated to increase the frequency of $CD4^+CD25^{hi}FOXP3^+$ cells after *in vitro* stimulation with GAD_{65} at 21 and 30 months after treatment, and a GAD_{65}-induced increase of $CD4^+CD25^{hi}FOXP3^+$ cells was accompanied by secretion of Th2 and regulatory cytokines [234]. Additionally, high titers of GAD autoantibodies (GADA) at baseline were related to a better clinical outcome in terms of C-peptide preservation [244]. Analysis of *in vitro* stimulated cytokine secretion and FOXP3 mRNA revealed secretion of IL-5 and IL-13 by peripheral blood mononuclear cells (PBMC), as well as increased expression of FOXP3 mRNA one month after the first injection. A broad range of cytokines were secreted by PBMC isolated at 3 and 9 months, but only Th2-associated cytokines and FOXP3 mRNA increased continuously [245]. Immunological studies at the 4-year follow-up demonstrated induction of memory T cells and T cell activation accompanied by secretion of Th1, Th2 and regulatory cytokines upon antigen stimulation *in vitro* [246].

Recent phase II and III trials

Two phase III trials with identical design were initiated, one in Europe and one in the US, following the promising results of the Swedish phase II trial. Additional phase II trials were also initiated, one intervention trial in the US run by the TrialNet consortium, and a Swedish prevention trial evaluating the safety and efficacy of GAD-alum in prevention of T1D.

The phase III trials were randomized, placebo-controlled, double-blind studies that aimed to include 320 GADA positive patients with T1D with less than 3 months disease duration, aged 10-20 years [247]. Patients were randomized to either of 3 treatment arms, receiving 4 doses of GAD-alum, 2 doses of GAD-alum followed by 2 doses of placebo, or 4 doses of placebo. Subcutaneous injections were administered at baseline and after 1, 3 and 9 months. Mixed-meal tolerance test was performed 6 times during the 30 month study period, and the primary endpoint was change in stimulated C-peptide between baseline and 15 months. The European trial included 334 patients. C-peptide levels were found to decline at similar rates in all three treatment arms, and there were no differences in glycated hemoglobin, insulin requirement or the rate of hypoglycemia compared to placebo. As the trial failed to achieve primary endpoint, it was immediately closed at 15 months, and only a minority of the participants completed the

21 and 30 month follow-ups. Preplanned exploratory subgroup analysis revealed significant effects on preservation of C-peptide secretion in four subgroups; 1) males, 2) patients with Tanner puberty stage 2 or 3 at baseline, 3) patients with baseline insulin dose of 0.398-0.605 IU/Day/kg, and 4) patients from non-Nordic countries.

The American phase III trial had not yet reached primary endpoint when the European trial was cancelled, and switched focus to instead collect safety data for at least 6 months following the last injection.

The phase II trial initiated by TrialNet included 145 GADA-positive patients aged 3-45 years with disease duration of less than 100 days. Patients were randomized to three arms like in the phase III trials, but the 4-dose group was replaced with a 3-dose group. The study failed to reach its primary endpoint; neither 3 nor 2 doses of GAD-alum had an effect on preservation of insulin secretion compared to placebo [248].

The Swedish phase II prevention trial of GAD-alum includes 50 children aged 4-18 years who are GADA-positive and positive for at least one additional T1D-related autoantibody and have not yet been diagnosed with T1D [249]. The participants are randomized to receive either 2 doses of 20µg of GAD-alum or 2 doses of placebo at baseline and 1 month, and will be monitored every 3 months for 5 years. The primary aim is to evaluate safety of GAD-alum treatment in healthy children and the secondary aim is to find out whether GAD-alum injections may prevent onset of T1D in at-risk children. No serious adverse events have been reported thus far and two children have developed T1D.

An intervention trial evaluating safety and effects of treatment with GAD-alum in combination with vitamin D and ibuprofen on residual insulin secretion (DIABGAD) is also currently recruiting participants. The hypothesis is that vitamin D will have positive effects on the immune system in conjunction with an anti-inflammatory effect of ibuprofen, which might increase the efficacy of GAD-alum treatment.

AIMS AND HYPOTHESIS

The general aim of this thesis was to study immune regulation in T1D and pre-diabetes, and the involvement of Treg in the immunomodulatory effect of GAD-alum treatment. It was hypothesized that GAD-alum treatment could induce antigen-specific Treg or improve the suppressive function of Treg in patients with T1D, which could contribute to increased tolerance to β-cell antigens, and improved clinical outcome.

Specific aims:

Paper I: To analyze and compare Treg frequencies in healthy, diabetic and at-risk subjects, to clarify the role of Treg in the progression to clinical T1D.

Paper II: To investigate whether an increase in cells with a Treg phenotype persisted 4 years after GAD-alum treatment, and to determine whether GAD-alum treatment affected the suppressive capacity of Treg.

Paper III: To evaluate the immunological effects of GAD-alum treatment and additional injections in patients with recent onset T1D, specifically the effects on Treg phenotype and frequencies, as well as antigen specific T cell frequencies.

Paper IV: To determine whether common polymorphisms in genes encoding proteins of the NALP3 inflammasome are associated with T1D, and whether these polymorphisms can affect clinical parameters or the outcome of GAD-alum treatment in children with T1D.

MATERIALS AND METHODS

Study populations

ABIS study (Paper I)

ABIS is short for All Babies in Southeast Sweden (Alla Barn i Sydöstra Sverige). It was initiated by Johnny Ludvigsson at the division of pediatrics, faculty of health sciences of Linköping University, and it was originally designed as a prospective study of T1D, allergy and autoimmune diseases in the Swedish population.

All mothers of children born in the southeast region of Sweden between October 1997 and 1999 were invited to participate in the study, and a total of 17055 children agreed to participate. Some children were HLA-typed for HLA-DR3, DR4, DQ2, DQ6 and DQ8. T1D associated autoantibodies GADA, IA-2A and IAA were analyzed in all children from whom at least one serum or whole blood sample was obtained at 1, 2.5 or 5 years of age, as described by Lynch *et al* and Larsson *et al* [250-251].

Using autoantibody titers at all three follow-ups, children were divided into groups with varying risk of developing T1D. Thus, paper I includes 9 children participating in the 8-year follow up of the ABIS study with autoantibodies against insulin, GAD, or IA-2 in the 95th percentile or higher at 2.5 and/or 5 years of age, as well as a group of 9 ABIS participants with no known risk for T1D, allergy or autoimmune disease.

Diabetes patients (Papers I and IV)

Patients diagnosed with T1D attending the pediatric clinic at Linköping University Hospital were included in papers I and IV. These patients were between 2-17 years old at onset, and were asked to give one additional blood sample for research purposes at each regular visit to the clinic. Informed consent was obtained from the parents and the child. Diagnosis was based on the American Diabetes Association criteria for diagnosis and classification of diabetes (i.e. casual plasma glucose>11.1 mmol/L or a fasting plasma glucose>7.0 mmol/L and symptoms of polyuria, polydipsia and weight loss) [252]. Paper I included 14 patients aged 10 (3-17) years old, 3 months after disease onset. Demographics of patients with T1D included in Paper IV are given in Table I.

Table I. Demographics of patients with T1D included in Paper IV.

Characteristic	**Males n=81**	**Females n=100**
Age at onset (range)	10.3 (2-17)	10 (2-17)
Fasting C-peptide at diagnosis (nmol/l)	0.35	0.37
Stimulated C-peptide 3 months after diagnosis (nmol/l)	0.89	0.74
Autoantibodies (%)	n=33	n=40
Negative for all tested	1 (3)	1 (2.5)
GADA	4 (12.1)	4 (10)
IAA	2 (6.1)	1 (2.5)
IA-2A	6 (18.2)	5 (12.5)
GADA + IAA	1 (3)	2 (5)
GADA + IA-2A	9 (27.3)	18 (45)
IAA + IA-2A	3 (9.1)	3 (7.5)
GADA + IAA + IA-2A	7 (21.2)	6 (15)

Age at onset values are means (range). Autoantibody values are number of patients positive for the given antibody.

GAD-alum phase II trial (Papers II and IV)

The GAD-alum phase II trial was a randomized, double blind, placebo-controlled clinical trial of recombinant human GAD_{65} formulated in aluminium hydroxide [242]. The trial included 70 Swedish patients who were randomized into two groups. Patients received either two injections of 20µg of GAD-alum (n=35) or two injections of placebo (alum alone; n=35) one month apart. Patients were recruited within 18 months after receiving the diagnosis of diabetes and had fasting C-peptide levels above 0.1 nmol per liter (0.3 ng per milliliter) and GAD autoantibodies. They underwent mixed-meal tolerance tests to determine stimulated C-peptide levels at the time of the first injection and at months 3, 9, 15, 21, 30 and 48 (Fig 6). One patient randomized to the placebo group was withdrawn from the study after one week due to mononucleosis.

The 48-month follow up was initiated separately in 2009, and 59 of the 70 original participants agreed to participate (29 GAD-alum, 30 placebo). Baseline characteristics for patients participating in the 4-year follow-up are given in Table II.

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

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

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

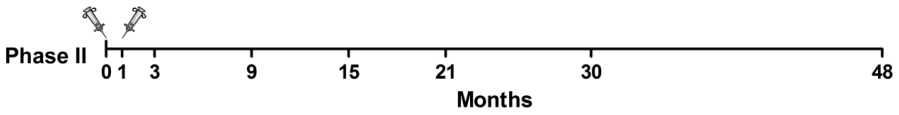

Figure 6. *Overview of sample collection in the GAD-alum phase II trial. Injections of GAD-alum or placebo were administered on day 0 and 1 month, samples were collected at the indicated time points, with the 4-year follow-up at 48 months.*

GAD-alum phase III trial (Paper III)

The phase III trial of GAD-alum included 334 patients from several countries in Europe (Finland, France, Germany, Italy, the Netherlands, Slovenia, Spain, Sweden and the United Kingdom), 148 of which were Swedes. Due to findings in the phase II trial indicating that the best clinical response to treatment was achieved in patients with T1D duration of less than 6 months, the inclusion criteria were changed to only include patients with T1D duration of 3 months or less. In addition to confirming the efficacy and safety of GAD-alum in T1D, the trial was designed to evaluate the effect of 2 additional booster injections. The trial thus

included three treatment arms, one receiving 4 injections of GAD-alum at 0, 1, 3 and 9 months, one receiving 2 injections of GAD-alum followed by 2 injections of placebo and another receiving 4 doses of placebo (Fig 7). These groups will be referred to as the 4-dose (4D), 2-dose (2D) and placebo group in this thesis. Of the Swedish patients, 49 were randomized to the 4D group, 49 to the 2D group and 50 to the placebo group. Baseline characteristics for the Swedish cohort are given in Table III.

Table III. Baseline characteristics according to study group in the phase III trial. Characteristics are given for the Swedish cohort *(Paper III)*.

	Swedish cohort		
Characteristic	**4D n=49**	**2D n=49**	**Placebo n=50**
Age (years)	13.3±2.1	13.3±2.2	13.4±2.5
Days since diagnosis	74.6±20.3	73.3±20.0	71.0±20.6
Gender distribution, n (%)			
Female	25 (51)	26 (53)	20 (40)
Male	24 (49)	23 (47)	30 (60)
HLA risk classification, n (%)			
very high	14 (29)	18 (37)	16 (32)
High	21 (44)	23 (47)	23 (46)
Moderate	8 (17)	4 (8)	8 (16)
Low	5 (10)	4 (8)	3 (6)
Tanner puberty stage, n (%)			
1	6 (12)	10 (20)	5 (10)
2+3	17 (35)	12 (25)	16 (32)
4+5	26 (53)	27 (55)	29 (58)
C-peptide (nmol/l)			
Fasting C-peptide	0.30±0.17	0.25±0.11	0.26±0.14
Stimulated C-peptide AUC	0.71±0.35	0.66±0.26	0.64±0.24
Glycated hemoglobin (%)	6.84±0.65	6.81±0.91	6.87±1.04
Insulin dose (IU/Day/kg)	0.59±0.29	0.62±0.31	0.55±0.23
Fasting plasma glucose (mmol/l)	6.28±2.16	5.95±1.74	5.69±1.19
Median GADA (Units/ml)	204	312	190

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. 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.

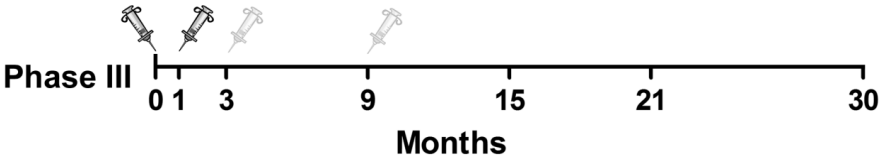

Figure 7. *Overview of sample collection in the GAD-alum phase III trial. Injections were administered at day 0, 1, 3 and 9 months. The 2D group received GAD-alum at day 0 and 1 month, and placebo at 3 and 9 months. Samples were collected at all time points indicated.*

Healthy individuals (Paper IV)

A biobank of 806 healthy individuals selected randomly from a population register in the southeast region of Sweden was established by Professor Peter Söderkvist between 1998 and 2000. This population has been used as a reference in studies of SNP distribution in Crohn's disease [253], colorectal cancer [254], sporadic malignant melanoma [255] and inflammatory disease [195].

PBMC isolation (Papers I, II and III)

PBMC were isolated from blood samples by Ficoll (Pharmacia Biotech) gradient centrifugation at 400G for 30 minutes within 24 h of collection. Cells at interface were harvested and washed three times in RPMI 1640 (Gibco) supplemented with 2% fetal calf serum. Cells were either washed in PBS 0.1% BSA and stained for flow cytometry analysis (paper I), immediately subjected to *in vitro* antigen stimulation or frozen down cryogenically for later use (papers II and III).

In vitro antigen stimulation (Papers II and III)

One million PBMC were cultured in 1ml AIM-V medium supplemented with 20µM β-mercaptoethanol in 5ml polypropylene tubes for 7 days at 37°C, 5% CO_2, with or without 5µg of recombinant human GAD_{65} (Diamyd Medical).

Flow cytometry

Flow cytometry is in essence a technique to count cells or other particles in suspension, several thousand per second. Cells are forced to pass an interrogation point one cell at a time through the establishment of a laminar flow of sheath fluid. The Bernoulli effect created by different pressures in the center and edges of the stream forces the cells to line up in the center of the stream, and thus data from each single cell can be collected.

At the interrogation point, cells pass one or more lasers in a cuvette. The cells will interfere with the laser light passing through it, and scatter the laser light. This is commonly measured as two parameters providing approximate data on cell size and granularity. Forward scatter (FSC) is measured at small angles from the incident laser beam and is affected by size, but also refractive index of cells. Apart from providing a rough estimation of cell size, this can also help discriminate "dead" cells, because their refractive index is lower and they will thus produce smaller FSC signals. Side scatter (SSC) is measured at a 90° angle from the incident laser beam, and is strongly affected by granularity of the measured cell. This allows discrimination between lymphocytes with a uniform cytoplasm and monocytes with more granular structures.

Cells are commonly labeled with monoclonal antibodies to surface antigens. The antibodies are in turn conjugated with fluorescent molecules, or fluorochromes. The wavelengths of laser lines in a cytometer match the excitation maximum of several available fluorochromes, giving rise to fluorescence in cells that have bound conjugated antibodies. Excited fluorochromes emit light at longer wavelengths than the light which excited it; this is called the Stokes shift. Like SSC, emitted fluorescence is measured at a 90° angle from the incident light. Emitted light of different wavelengths is separated in a system of dichroic mirrors and directed to separate detectors. Before reaching the detector, the beam will pass a bandpass filter which transmits light of a narrow range of wavelengths. This principle for how different photomultiplier tube detectors measure different fluorochromes is illustrated in Figure 8. Each event will give rise to a pulse peak in each detector, with a height, width and area. The area is most commonly used to measure fluorescence intensity in each channel.

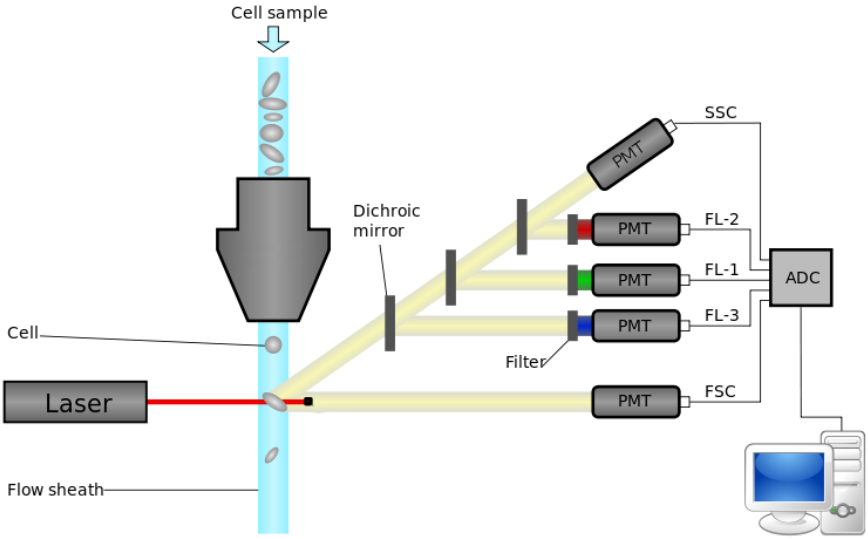

Figure 8. *Schematic illustration of flow cytometry. PMT = photomultiplier tube, ADC = Analog-to-digital converter, FL = Fluorescence detectors. Image adapted from Wikipedia user Kierano (http://en.wikipedia.org/wiki/File:Cytometer.svg, accessed 2013-09-14), under the creative commons license.*

Fluorochromes often have broad emission spectra, or a spectrum with secondary peaks of emission. This leads to a phenomenon called spectral overlap, where one fluorochrome emits light that will be registered by a detector intended for the emission maximum of another fluorochrome. A green fluorochrome might also emit yellow light, for example, and it would appear that cells stained with the green fluorochrome also bound some antibody conjugated with yellow fluorochrome, even though they did not. Luckily, the spillover is always directly proportional to the signal measured in the "proper" detector, and compensation matrices can be employed when fluorochromes with overlapping spectra are used, where a set percentage of the signal in one channel will be deducted from another.

Flow cytometry thus allows for characterization of cells based on morphology, as well as expression of surface markers and intracellular proteins or DNA content. It is a very powerful tool to examine frequencies of different cell types in a sample *ex vivo*, or to assess the expression of markers on cells that have been cultured under different conditions *in vitro*. A list of monoclonal antibodies and other staining reagents used in this thesis is presented in Table IV.

Table IV. CD markers and monoclonal antibodies.

Marker	Function	Clones/ Fluorochromes	Used in Paper
CD3	Part of T cell receptor	OKT3	II
CD4	Expressed by T helper cells	SK3 PerCP	I
	Interacts with HLA class II	RPA-T4 Alexa 700	II, III
		RPA-T4 Pacific Blue	II
CD8	Expressed by cytotocix T cells	RPA-T8 Alexa 488	III
	Interacts with HLA class I	RPA-T8 APC	III
CD25	IL-2 receptor alpha chain	2A3 APC	I
		M-A251 APC-Cy7	II, III
		BC96 APC	II
CD27	TNF-receptor superfamily	M-T271 FITC	I
CD28	Co-stimulation for T cell receptor	CD28.2	II
CD39	Ectonucleotidase	A1 FITC	II
CD45RA	Marker of naïve T cells	HI100 PE-Cy5	III
CD45RO	Marker of memory T cells	UCHL1 APC	III
CD101	V7 antigen, Ig superfamily	BB27 APC	II
CD127	IL-7 receptor subunit alpha	eBioRDR5 FITC	II
		eBioRDR5 PE-Cy7	II, III
CTLA-4	Negative co-stimulator	BNI3 PE	I
		BNI3 APC	III
FOXP3	Transcription factor	PCH101 PE	I, II, III
HLA-DR	Antigen presentation	L243 Alexa 488	III
CFSE	Cell tracking dye		III
Tetramers	Binds specific T cell receptors	PE	III

Markers stained in flow cytometry or utilized in activation of T cells through monoclonal antibodies. CFSE is detected in FL1/FITC channel, Tetramers were HLA-DR301, DR401 or DR404 restricted and loaded with 5 different peptides of GAD_{65} in total.

Four-color flow cytometry (Paper I)

Flow cytometry experiments for paper I were performed using a FACSCalibur flow cytometer, measuring 6 parameters, 4 fluorochromes plus scatter. Briefly, *ex vivo* PBMC separated immediately after sampling were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CTLA-4, phycoerythrin (PE)-conjugated anti-FOXP3, peridin-clorophyll (PerCP)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD25 and FITC-conjugated CD27 antibodies. Cells were incubated with CD4 and CD25 antibodies prior to fixation/permeabilization and FOXP3 and CTLA-4 antibodies after permeabilization. Five hundred thousand lymphocytes were collected from each tube. Data was acquired and analyzed using Cellquest pro software (Becton Dickinson).

Seven-color flow cytometry (Papers II and III)

PBMC were incubated with and without 5µg of GAD_{65} for 7 days before staining in papers II and III.

In paper II, one million cells were stained with FITC-conjugated anti-CD39, PE-conjugated anti-FOXP3, APC-conjugated anti-CD101, PE-cyanine 7 (PE-Cy7)-conjugated anti-CD127, APC-Cy7-conjugated anti-CD25 and alexa fluor 700-conjugated anti-CD4 antibodies, as described for four-color FACS.

In paper III, three different staining panels were used. The first panel, designed to assess the frequency and phenotype of Treg, included HLA-DR-alexa fluor 488, FOXP3-PE, CD45RA-PE-Cy5, CTLA-4-APC, CD4-alexa fluor 700, CD127-PE-Cy7, CD25-APC-Cy7. The second panel was desgined to monitor phenotype and frequencies of GAD_{65}-specific T cells and substituted GAD_{65} peptide loaded HLA class II tetramers labeled with PE and CD45RO-APC and CD8-alexa fluor 488 for FOXP3, CTLA-4 and HLA-DR antibodies. Tetramers bind T cells with a TCR specific for the peptide loaded onto the tetramer. This allows staining of cells specific for a certain antigen. Tetramers preferentially bind T cells with a TCR with high avidity for the antigen and this may mean that Treg, which commonly have a lower avidity TCR-HLA interaction, are not stained as well as cells with a high avidity TCR. Regardless, tetramer staining remains the best method for identifying antigen specific T cells using flow cytometry.

The final panel included the dye CFSE (see: proliferation assays) to determine the proliferative response of different populations of lymphocytes to GAD_{65} antigen stimulation. The CFSE-panel also included the dye 7-AAD which stains dead cells, CD8-APC, CD4-alexa fluor 700, CD127-PE-Cy7 and CD25-APC-Cy7.

Cells were acquired on a FACSAria and data was analyzed using Kaluza software. Two hundred thousand lymphocytes were acquired from each tube. Due to the varying numbers of patients included in separate analyses of flow cytometric data from the phase III trial, the number of available samples for each staining and group is given in Table V.

Table V. Numbers of individuals included in analysis of expression of each marker by group and follow-up in the phase III trial.

	CD25/CD127/CD45RO			FOXP3/CTLA-4/HLA-DR			CD45RA			CFSE			Tetramer		
Month	**4D**	**2D**	**Placebo**	**4D**	**2D**	**Placebo**	**4D**	**2D**	**Placebo**	**4D**	**2D**	**Placebo**	**4D**	**2D**	**Placebo**
0	14	16	14	16	17	16	N/A	N/A	N/A	9	7	8	N/A	N/A	N/A
1	17	15	16	14	16	18	N/A	N/A	N/A	8	3	7	0	2	3
3	20	20	19	20	22	18	N/A	N/A	N/A	11	10	8	7	3	1
9	25	23	23	26	25	26	9	14	14	4	4	5	7	3	4
15	31	24	20	29	26	21	28	23	19	N/A	N/A	N/A	5	1	3
21	20	16	12	19	15	12	18	15	12	N/A	N/A	N/A	3	4	3

Number of individuals with complete staining for indicated markers at indicated follow-ups. 4D = 4 doses of GAD-alum, 2D = 2 doses of GAD-alum.

Analysis of flow cytometry data

The analysis of flow cytometry data involves a process called gating, in which events recorded by the flow cytometer are delineated into discrete populations by imposing cut-off values of fluorescence intensity in different channels. This process can be aided by the use of isotype controls or fluorescence minus one controls, which serve as negative controls for fluorescence intensity. Frequently a negative population is present in the sample itself, and sometimes gates are set subjectively because they define a population with higher expression of a given marker.

In paper I, events were first gated based on morphology, i.e. FSC and SSC, to define the lymphocyte population. The T cell gate was based on CD4 expression, the $CD25^{hi}$ gate was set to include $CD4^+$ cells with higher CD25 expression than $CD4^-CD25^+$ cells. FOXP3, CD27 and CTLA-4 gates were set according to isotype controls. Median Fluorescence Intensity (MFI) of FOXP3 was analyzed in paper I. The fluorescence intensity of a fluorochrome on each cell is proportional to the amount of expression of the target marker on that cell, and MFI can thus be used to relatively quantify protein expression per cell, as opposed to the frequency of cells that are considered positive for the marker.

In papers II and III, the lymphocyte and $CD4^+$ gates were set in a similar fashion. A gate discriminating cells with higher FSC and SSC was used to select GAD-induced cells. The $FOXP3^+$ gate was set using the $FOXP3^-$ population, since it had higher fluorescence intensity than the isotype control. The gates for $CD25^+CD127^{lo}$ and $CD25^+CD127^+$ cells were set subjectively based on examples in the literature [120]. The CD45RA/FOXP3 gates in paper III were based on the work of Miyara et al [118]. Gates for proliferating cells based on CFSE fluorescence were set so that less than 0.1% of cells in an unstimulated sample were classified as dividing. The $Tetramer^+$ gate was set so that 0.1% of cells were positive when stained with an irrelevant tetramer.

Cell sorting

Cells can be sorted utilizing flow cytometry in various ways. The objective is to separate cells with a desirable expression of surface markers from unwanted cells. On the FACSAria used in all sorting experiments in this thesis, the fluid stream containing cells passes through a small orifice called a nozzle, that can be between 70-130µm in diameter, which makes it hydrodynamically unstable, causing it to break up into a series of droplets. The droplet formation can be made stable if the stream is subjected to vibration, which is produced by a piezoelectric transducer acoustically coupled to the flow chamber on the FACSAria. Positive or negative voltage pulses are applied to the stream before droplet break-off, and when a droplet separates from the stream, it carries surface charge of the same polarity. The droplets then pass two near-parallel deflection plates charged with high positive or negative voltages, moving charged droplets toward the oppositely charged plate. By applying charge to droplets containing cells that fit the sorting criteria set up by the user, cells can thus be deflected from the main stream of droplets into collection tubes, or microtiter plates. This is illustrated in Figure 9.

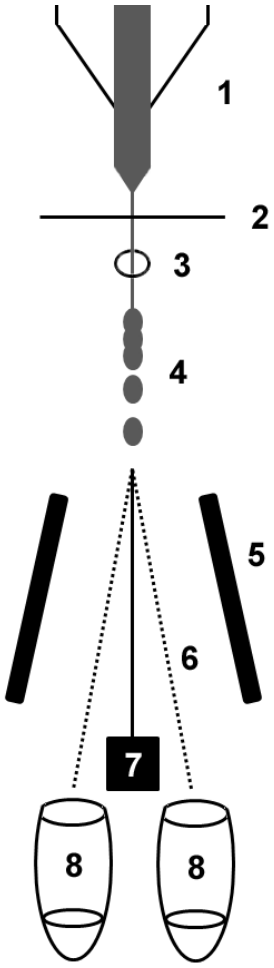

Figure 9. *Schematic illustration of fluorescence activated cell sorting on a FACSAria cell sorter.* ***1****. Cells are centered in a stream of sheath fluid in the flow cell.* ***2****. Centered cell stream passes the lasers at the interrogation point.* ***3****. Cells pass through a nozzle of variable size.* ***4****. Droplets of fluid break off the stream due to a vibration induced by a piezo element, a charge is applied to droplets based on the data acquired at the interrogation point.* ***5****. Droplets ideally containing one cell each pass an electromagnetic field established by deflection plates.* ***6****. Charged droplets are directed to either side in the field, creating side-streams.* ***7****. Uncharged droplets are directed to waste.* ***8****. Charged droplets are collected in tubes.*

The FACSAria uses a set of variables called masks to optimize yield and purity of the sorted cells. The masks set limits on how close a cell to be sorted can be to another event recorded by the cytometer. If the main concern is the purity of the sorted population, the masks are more strict, and the sorting of a cell that is close to another droplet containing an unwanted cell will be aborted. If a higher yield is desired, the masks are conversely less strict to maximize the number of cells sorted while risking contamination from neighboring droplets.

Treg and Teff sorting (Paper II)

Treg and Teff were sorted from PBMC samples thawed and allowed to rest for 24 hours in AIM-V medium after cryopreservation. PBMC were stained with CD4-Pacific Blue, CD127-FITC and CD25-APC. Lymphocytes were determined by FSC and SSC, $CD4^{+}CD25^{+}CD127^{lo/-}$ cells were sorted as Treg and $CD4^{+}CD25^{-}CD127^{+}$ cells were sorted as Teff, using a FACSAria equipped with a 100µm nozzle on the purity setting (Fig 10). 7-AAD was used to exclude dead cells. Sorted cells were collected in fetal calf serum-coated 5ml polypropylene tubes containing 1ml AIM-V medium supplemented with 10% human serum and kept at 4°C for the duration of sorting, and then immediately pelleted by centrifugation and resuspended in fresh AIM-V 10% human serum. They were allowed to rest for 2 hours before expansion was initiated (see: Treg and Teff expansion). A portion of cells were sorted and re-analyzed immediately, the frequency of Teff contaminating sorted Treg was generally 0.1% of sorted cells.

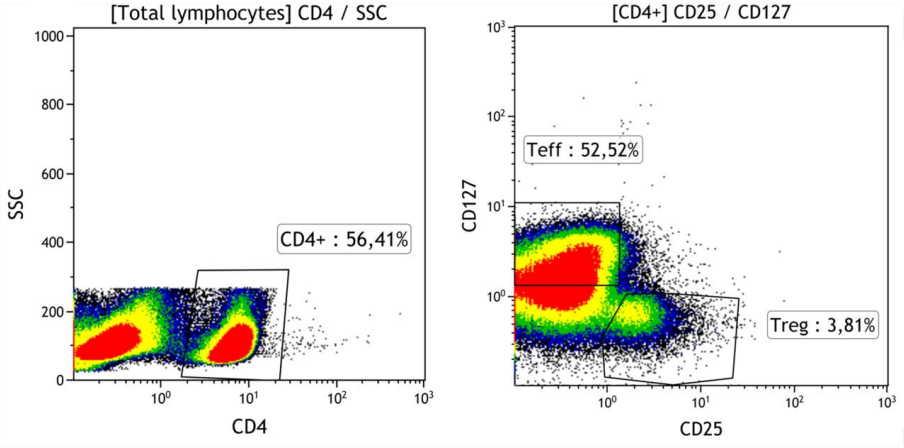

Figure 10. *Gates used to sort Treg and Teff. Left panel shows discrimination of CD4$^+$ T cells, right panel shows discrimination of Treg and Teff based on CD25 and CD127 expression. Representative frequencies of cells are indicated in plots. Right panel shows only CD4$^+$ cells.*

Treg and Teff expansion (Paper II)

Treg are a fairly rare cell type in peripheral blood, which is a problem when dealing with venous samples from very young children. It is desirable to draw blood from children as seldom as possible, and it is also frequently difficult to acquire large volumes of blood from a child. This significantly limits the source material and thus the number of Treg than can be sorted are in turn limited, to the point where performing even a single experiment with the sorted cells is sometimes ruled out. An expansion protocol was utilized to address this issue, providing a large number of cells for further *in vitro* experiments.

Sorted Treg were aliquoted into 96-well plates at 4x10^4 cells per well and stimulated with CD3/CD28 coated beads (Dynal) at 1:1 bead per cell ratio after having been allowed to rest for 2 hours. On day 2, 300U/ml of IL-2 (R&D) was added to Treg cultures. On day 5 and 7, Treg were washed in fresh AIM-V medium and resuspended in AIM-V 10% human serum, and fresh IL-2 was added. On day 9, Treg were restimulated as above, washed every other day and preserved cryogenically on day 15 of culture. This resulted in an approximately 1000-fold expansion of Treg, while maintaining lineage specificity as determined by FACS, gene expression and cytokine secretion analysis.

Teff were subjected to a similar expansion protocol, but were aliquoted into wells pre-coated with 10µg/ml anti-human CD3 antibody (OKT3, eBioscience) at $5x10^5$ cells per well. Anti-human CD28 antibody (CD28.2, eBioscience) was also added to these cultures at 1µg/ml, and IL-2 was added on day 2 and onwards as above at 30U/ml. Teff cultures were given fresh medium when necessary, but were never washed to remove all medium. Like Treg, Teff were cryogenically stored after 15 days of expansion, with an approximately 100-fold expansion in cell number.

Proliferation assays

^{3}H-Thymidine incorporation assay (Paper II)

Tritium is a β-emitting radioisotope of hydrogen with a half-life of approximately 12 years, and is very rare in nature. It replaces hydrogen atoms in ^{3}H-thymidine. Since thymidine is a component of DNA, cells incubated with ^{3}H-thymidine will incorporate it into their DNA upon replication before cell division. The β-emission is then measured using a liquid scintillation counter, and is proportional to the amount of cell division.

The proliferation assay in paper II was set up in duplicate U-bottom wells of $5x10^4$ Teff in a total volume of 200µl AIM-V 10% human serum containing 1U/ml IL-2. Treg were added in a dilution series of Treg:Teff ratios of 1:1, 1:2 … 1:16, Treg and Teff were also cultured separately. Cells were stimulated polyclonally using 5µg/ml soluble anti-CD3 and 1µg/ml anti-CD28 antibodies. Unstimulated Teff from patients and inter-assay control were included as negative controls. Cells were cultured for 3 days at 37°C, 5% CO_2, after which a portion of the culture medium was removed and replaced with ^{3}H thymidine-containing medium with an activity of 0.2µCi. Cells were harvested after 18 hours of incubation with ^{3}H-thymidine and thymidine incorporation was measured using a 1450 Wallac MicroBeta counter (PerkinElmer).

CFSE dilution (Paper III)

Carboxyfluorescein succinimidyl ester is a fluorescent dye that covalently binds to lysine residues on proteins. Cells are incubated with the non-fluorescent carboxyfluorescein diacetate succinimidyl ester CFDA-SE, which readily enters the cell by diffusion where the acetate groups are cleaved by esterases, forming CFSE. After an initial expulsion of the dye during the first 24 hours after incubation, CFSE is retained by cells and remains stable in non-dividing cells. Dividing cells will however dilute their CFSE content upon mitosis, facilitating tracking of cell divisions in an *in vitro* culture. While high concentrations of the dye are cytotoxic, low concentrations are tolerated fairly well. CFSE is excited at 488nm and its fluorescence can thus be measured by a flow cytometer. In combination with surface staining, the proliferation of discrete populations of cells in culture can be measured.

In paper III, 1 million PBMC were incubated with 1µM CFSE at 4°C for 10 minutes in PBS. The reaction was stopped with 1ml of human serum supplemented medium for 2 minutes. CFSE stained cells were then washed with PBS and cultured as described in the *in vitro* antigen stimulation section, and acquired after 7 days of culture.

Gene expression (Paper II)

RNA isolation

Total RNA from expanded Treg and Teff in paper II was isolated according to the RNeasy 96 vacuum/spin protocol (Qiagen). Isolated RNA yield was quantified by measuring absorbance at 260 nm using a spectrophotometer (Nanodrop). Nucleic acid purity was ensured with a 260/280 absorbance ratio of ~2.

PCR array

The polymerase chain reaction (PCR) array is an application using realtime PCR (described in Realtime PCR) to analyze the expression of several target genes. This particular assay uses SYBR green dye, which binds to double stranded DNA, and target-specific primers in each well which allows for gene-specific exponential increase in fluorescence with each amplification cycle.

In paper II, the expression of 18 target genes in expanded Treg and Teff was analyzed using a customized Human Gene RT^2 profiler™ PCR array (SABiosciences). Isolated RNA was transcribed into complementary DNA (cDNA) using RT^2 First Strand Kit (SABiosciences) and combined with RT^2 SYBR® Green/ROX™ qPCR Master Mix. Gene-specific primer sets were pre-dispensed in 96-well plates. ABI Prism 7900HT was used for sequence detection, and sequence detection systems (SDS) version 2.3 (Applied Biosystems) was used to calculate threshold cycle (C_t) values. Raw C_t values were normalized to HPRT1, found by NormFinder to be the most stable housekeeping gene among the ones included in the array. For each gene, the expression was relatively quantified to the sample with the lowest expression.

Genotyping

DNA isolation (Papers III and IV)

DNA from diabetic patients and participants in the GAD-alum phase II trial for the TaqMan genotyping assay described below was isolated using a Maxwell 16 (Promega) automated robot for DNA, RNA or protein extraction, using pre-made cartridges with appropriate reagents.

A spin column kit, EZNA blood DNA kit (Omega Bio-Tek), was used to purify DNA from whole blood for the DR3/4 subtyping assay used to determine HLA-DR genotypes in paper III, which is described below.

Isolated DNA was quantified by measuring absorbance at 260 nm using a spectrophotometer (Nanodrop). Nucleic acid purity was ensured with a 260/280 absorbance ratio of ~1.8.

Realtime PCR

Realtime PCR is a polymerase chain reaction where the amount of product is measured after each cycle. Primers are used to amplify a selected region like in standard PCR. In addition, an oligonucleotide probe is used, which is conjugated with a fluorochrome at the 5' end and a quencher at the 3' end. When a laser hits the sample the fluorochrome will be excited, but because of the close proximity to the quencher, the quencher will absorb the energy that would normally be converted into an emitted photon. However, when the DNA fragment that

has bound the probe is transcribed by a certain nuclease, the quencher and fluorescent probe are cleaved, and the fluorochrome is free to emit fluorescence, producing a signal proportional to the amount of copies of the target sequence during each cycle.

TaqMan genotyping assay (Paper IV)

A TaqMan genotyping assay uses two probes, each specific for one allele, that anneal to the polymorphic site under conditions where the probes cannot anneal with a mismatch. Primers and probes were added to the template DNA along with a polymerase-containing master mix and thermal cycling carried out on an ABI Prism 7900HT (Applied Biosystems). A pre-read was performed to determine the initial copy numbers of alleles in the genomic DNA. After thermal cycling, a post-read was performed and the alleles determined based on the fluorescence intensity of each probe using SDS version 2.3 (Applied Biosystems).

DR3/4 subtyping assay (Paper III)

A realtime PCR based method using TaqMan probes for determining DRB1*04 alleles as well as determining presence of DR3 alleles was used in paper III [256]. Pan-DR4 primers and probe were designed to recognize all DR4 alleles. DR4 subtyping reactions were designed to use a single pair of DR4 group-specific primers together with 9 different FAM or VIC labeled probes in 5 separate reactions for each sample, collectively identifying the major polymorphisms in DRB1. In combination, these probes distinguish between most DR4 alleles. Thermal cycling was carried out on an ABI Prism 7900HT (Applied Biosystems). Thresholds were set at the exponential phase of the PCR reaction, typically at 0.05, and the cycle during which each reaction reached its C_t was determined using SDS version 2.3 (Applied Biosystems).

A pre-made sheet for Microsoft Excel designed for the specific assay, supplied by Gersuk *et al*, was used to translate realtime PCR results into DR4 subtypes [256]. In essence, cut-off values of C_t were used to determine positivity, where the reaction in question should have a C_t of 34 or less, while other reactions should have a C_t of 40 or more.

Luminex (Papers II and III)

Luminex is a flow cytometry-based technique utilizing microspheres filled with two fluorochromes and with covalently bound capture antibodies on the surface. Up to 100 sets of beads can be distinguished by alteration of the ratio of the two fluorochromes contained in the microspheres. Beads capture different analytes depending on the bound capture antibody. Biotinylated detection antibodies then bind the analyte captured on the beads, and streptavidin-PE is bound to biotin. The PE fluorescence for each bead set is used to quantify the analyte bound by each set of beads using standards with known concentrations of the analyte.

For the cytokine measurements in paper II and III, acquisition conditions were set with a minimum of 100 beads per bead set. Median fluorescence intensities were analyzed using Star Station software. A five-parameter curve fit was applied to each standard curve to obtain concentrations of each analyte. A Bio-Plex cytokine panel (Bio-Rad) was used according to the manufacturer's instructions.

C-peptide measurement (Papers II, III and IV)

In patients participating in the GAD-alum phase II trial, serum C-peptide concentration was measured at 0, 30, 60, 90 and 120 minutes after a mixed meal tolerance test (Sustacal/Boost) of 6ml/kg body weight using a time-resolved fluoroimmunoassay (AutoDELFIA^{TM} C-peptide kit, Wallac), as described [242]. Results were validated using high, medium and low level C-peptide controls (Immulite, DPC). The same methodology was used to determine C-peptide levels in patients with T1D included in Paper IV.

In the GAD-alum phase III trial, all C-peptide analyses were performed by BARC Laboratories (Ghent, Belgium). C-peptide quantification was performed using an Immulite 2000 C-peptide kit and analyzer with calibration standards based on the World Health Organization's National Institute for Biological Standards International Reference Reagent standards (product number, 84/510) [247].

The participants in the GAD-alum phase II trial were stratified into groups of responders, intermediate responders and non-responders based on changes in stimulated C-peptide area under the curve (AUC) from baseline to 15 months. Patients who lost less than 10% AUC were classified as responders, patients who lost between 10 and 65% as intermediate

responders and those who lost more than 65% AUC were classified as non-responders. Change in C-peptide AUC was also considered the main study outcome, and was the measurement of clinical efficacy in the trial.

Statistics

Nonparametric tests corrected for ties were used for all statistical testing in papers I-III since data was rarely normally distributed, as determined by D'Agostino and Pearson omnibus normality test. Mann-Whitney U-test was used to test two groups of unpaired samples. Wilcoxon signed ranks test was used for paired analysis of matched samples in two groups. Linear regression was used to compare slope and Y-intercept of suppression curves in paper II, and correlations were tested with Spearman's rank correlation coefficient test. Statistical testing was carried out using GraphPad Prism 5.04 (GraphPad Software Inc.) in papers I-III. Probability levels <0.05 were considered to be statistically significant.

Statistical analyses were performed with linear and logistic regression analysis in paper IV. Linear regression was used for continuous dependent variables and logistic regression was used to discriminate between cases and controls. The results from the logistic regression are presented with Odds Ratios (OR) and 95% confidence intervals. P-values were Bonferroni adjusted. The statistical package used to analyze results was STATA v11.1 (Stata Corp.).

Ethics

All studies were approved by the regional research ethics committee at the faculty of health sciences, Linköping University, Sweden. In paper I, via proxy consent was obtained for participating children, and presumed consent was obtained from parents upon completing and submitting questionnaires on entering the ABIS study. Written informed consent was obtained from all participants in the GAD-alum trials, and from the parents of patients younger than 18 years, in accordance with the declaration of Helsinki.

RESULTS AND DISCUSSION

Treg phenotype in healthy, at-risk and diabetic children

At the time this project was initiated, there were contradictory reports in the literature regarding frequencies of peripheral Treg in T1D. While there was support for Treg having a central role in autoimmunity and T1D from animal studies [257], some early experiments in humans reported reduced frequencies of Treg [161], while others could not replicate this finding, albeit in slightly different settings [93,162,164]. The contrasting findings were thought to be caused by different age-matching of control groups [258]. However, little was known about Treg frequencies in at-risk or pre-diabetic children and in young patients with T1D. Based on these previous results we hypothesized that Treg frequencies would be lower in children with T1D, and that they could be involved in progression to T1D if they were also lower in at-risk children.

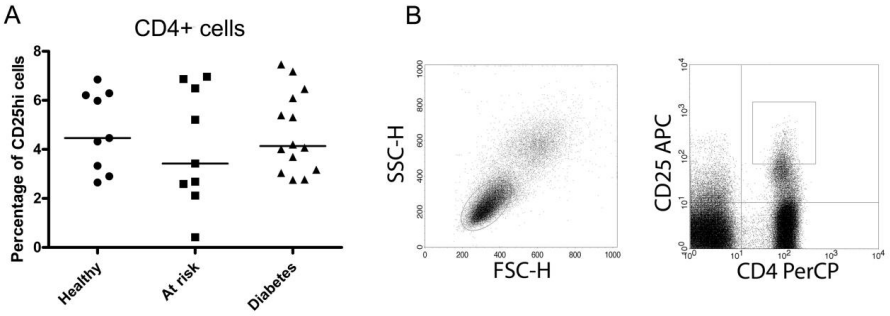

***Figure 11. A**. Percentage of $CD4^+CD25^{hi}$ cells in peripheral blood of healthy children, at-risk children and patients with recent onset T1D. **B**. Gates used to discriminate lymphocytes and $CD4^+CD25^{hi}$ cells.*

Increased FOXP3 and CTLA-4 expression in at-risk and recent-onset diabetic children

We could detect no difference in the frequency of $CD4^+CD25^{hi}$ cells between healthy, at-risk and diabetic children, which was in agreement with several previously published studies (Fig 11) [93,162,164], although these previous results were obtained using adult control populations and/or patients with long-standing T1D. However, contrary to expectations, we found that

$CD4^+CD25^{hi}FOXP3^+$ and CTLA-4^+ cells were more frequent in children with T1D compared to healthy children (Fig 12). At-risk children also had higher frequencies of $CD4^+CD25^{hi}$CTLA-4^+ cells compared to healthy children (Fig 12). These differences remained significant when analyzing total $CD4^+$ cells. At the time these experiments were performed, FOXP3 was considered the gold standard marker for the Treg lineage, but shortly after it was discovered that FOXP3 was upregulated in activated conventional T cells. The fact that activated Teff can express FOXP3 causes concern that our finding might represent increased numbers of activated Teff rather than Treg [110-112,259-260], and makes it harder to draw a clear conclusion from this finding.

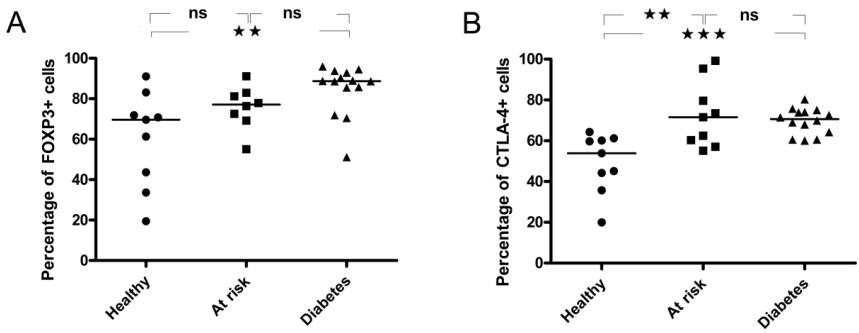

***Figure 12. A**. Percentage of $CD4^+CD25^{hi}$ cells expressing FOXP3. **B**. Percentage of $CD4^+CD25^{hi}$ cells expressing CTLA-4.ns = not significant, ** = p<0.01, *** = p<0.001.*

Treg of T1D patients express higher levels of FOXP3 protein

We also found that the MFI of FOXP3 in $CD4^+CD25^{hi}$ cells was higher in patients with T1D compared to healthy children, and that FOXP3 MFI correlated with $FOXP3^+$ cell frequency in children with T1D, but not in healthy children (Fig 13). The MFI of a marker correlates with its expression on individual cells in flow cytometry, thus this reflects the amount of FOXP3 expressed by individual cells in contrast to the frequency of FOXP3 expressing cells. A correlation between $FOXP3^+$ cell frequency and MFI had been reported previously, in patients with multiple slecoris and healthy subjects [261]. The same study found a weak but significant correlation between FOXP3 MFI and suppressive capacity of $CD25^{hi}$ cells, which supports the idea that the patients with T1D included in our study has a functional peripheral Treg population despite having an autoimmune disease. Though this seems counterintuitive,

several studies have shown that deficient Treg-mediated suppression in T1D is intrinsic to the Teff population [163,169]. It can thus be hypothesized that patients with T1D have a functional Treg population that is unable to suppress autoreactive Teff due to a resistance to regulation intrinsic to the Teff.

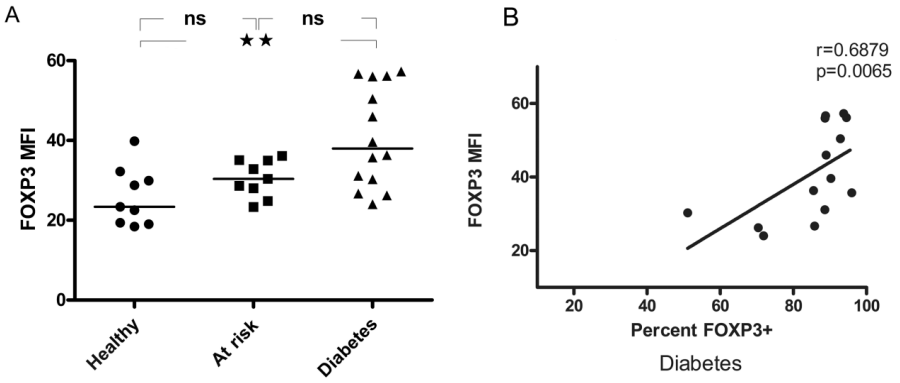

***Figure 13. A**. Median Fluorescence Intensity of FOXP3 on $CD4^{+}CD25^{hi}$ cells. **B**. Correlation of FOXP3 MFI and frequency of $CD4^{+}CD25^{hi}FOXP3^{+}$ cells in patients with recent-onset T1D. ns = not significant, ** = $p<0.01$.*

CD27 does not define Treg in peripheral blood in T1D

CD27 was used to define Treg in inflamed synovia in conjunction with CD25, and expression of CD27 was reported to delineate a more suppressive subset of Treg [262-263]. We found that children with T1D had higher frequencies of $CD4^{+}CD25^{hi}CD27^{+}$ cells than healthy children. The difference was however very small, ~95% in healthy children compared to ~97% in children with T1D. In addition, the frequency of CD27 among $CD4^{+}$ cells was comparable to that obtained among $CD25^{hi}$ cells. Thus we concluded that CD27 could not be used to define Treg in T1D. This result contrasted with previous findings that argued for the use of CD27 as a marker for Treg [261-262], but was in agreement with previous findings reported by Duggleby *et al* from a study including healthy individuals and patients with reactive arthritis, coming to the same conclusion [264].

T cell frequency and phenotype after GAD-alum treatment

Changes in T cell frequencies and induction of tolerance to β-cell antigens were hypothesized to be involved in the mechanism of action of GAD-alum treatment. Treg frequencies were analyzed in the phase II trial, but a limited number of markers were used and analysis was only performed 21 and 30 months after treatment, whereas we had the opportunity to follow Treg frequencies using an extended panel of markers throughout the phase III trial and 4 years after treatment in the phase II trial. We were further interested in analyzing immunological effects of 4 doses of GAD-alum. The incubation time during GAD_{65} stimulation was increased from 18 hours to 7 days in an effort to limit the effect of activation-induced FOXP3 expression in conventional T cells. The immunological phenotyping performed in the phase III trial included further characterization of Treg as well as memory T cells, antigen-specific T cells and T cell proliferation on antigen recall. 148 Swedish patients participating in the phase II trial and randomized to placebo, 2 or 4 doses of GAD-alum were included in immunological experiments. The inclusion criteria of the phase III trial were different from the phase II trial to reflect findings that indicated clinical benefit mainly in patients with shorter disease duration.

Antigen recall induces cells with both Treg and Teff phenotypes

Using an expanded panel of markers in our flow cytometric analysis, immunological effects were observed after administration of 2 doses of GAD-alum, i.e. at 3 months after start of treatment. The frequency of $CD4^+CD25^+CD127^+$ Teff was increased significantly after 7 days incubation with GAD_{65} in both GAD-alum treated groups 3 months after treatment, persisting throughout the trial, while the frequency of $CD4^+CD25^{hi}CD127^{lo}$ Treg was increased at 3 and 9 months but not 15 or 21 months after treatment (Fig 14A-B). Like in the phase II trial, the increase in Teff frequency was more pronounced than the increase in Treg frequency. $CD4^+FOXP3^+$ frequency was increased at 3, 9 and 21 months in the 2D group, and at 9 and 21 months in the 4D group (Fig 14C). CTLA-4 expression was also increased after GAD_{65} stimulation at 3 and 9 months in both GAD-alum treated groups, but remained higher only in the 4D group at 15 and 21 months. GAD_{65} stimulation also induced expression of HLA-DR in both the 2D and 4D groups at 3, 9, 15 and 21 months. However, FOXP3, CTLA-4 and HLA-DR can all be expressed by activated T cells, and indeed we found that increased expression of these markers was common in the $CD4^+CD25^+CD127^+$ Teff population compared to the $CD4^+CD25^{hi}CD127^{lo}$ Treg population, in which FOXP3 expression was not significantly

different after GAD-stimulation (Fig 14D-E). Thus, while GAD_{65} stimulation increases the expression of these Treg-associated markers on total T cells, their expression is increased in both Treg and Teff subsets after antigen recall.

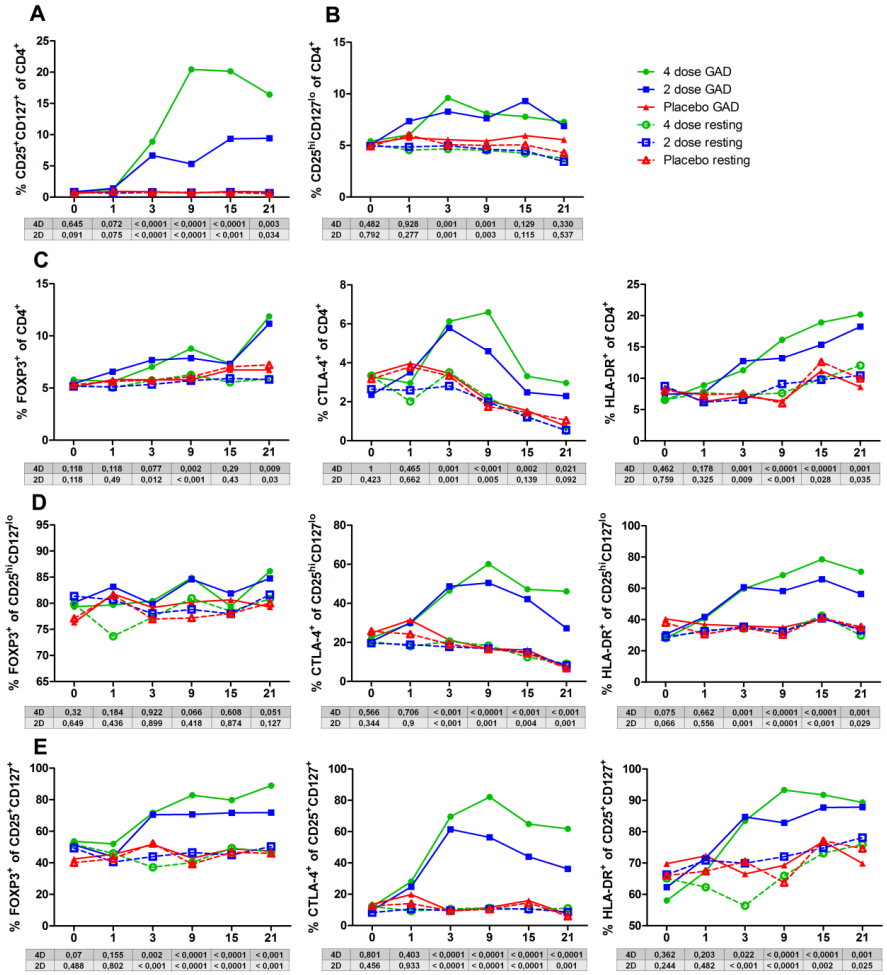

***Figure 14. A**. Frequencies of $CD4^+CD25^+CD127^+$ cells (Teff) in the 4D (green circle), 2D (blue square) and placebo group (red triangle) at each follow-up of the study period. Filled symbols indicate GAD_{65} stimulated experiments. **B**. Frequencies of $CD4^+CD25^{hi}CD127^{lo}$ cells (Treg). **C**. Frequencies of $CD4^+$ cells expressing FOXP3, CTLA-4 and HLA-DR are illustrated in left, center and right panel, respectively. **D**. Frequencies of $FOXP3^+$, $CTLA\text{-}4^+$ and $HLA\text{-}DR^+$ cells in the $CD25^{hi}CD127^{lo}$ Treg population. **E**. Frequencies of $FOXP3^+$, $CTLA\text{-}4^+$ and $HLA\text{-}DR^+$ cells in the $CD25^+CD127^+$ Teff population. P-values indicate comparisons of GAD_{65} stimulated cells from 4D and 2D groups compared to placebo.*

Treg frequency 4 years after GAD-alum treatment

In an extended 4-year follow-up of the phase II study, we aimed to determine whether a GAD_{65}-induced effect on Treg frequencies remained in patients who received GAD-alum. Our group had previously shown that GAD-alum treated patients with T1D upregulated $CD4^{+}CD25^{hi}FOXP3^{+}$ cells after 18h incubation with GAD_{65} 21 and 30 months after treatment [234]. In this follow-up we were able to better define Tregs. The addition of CD127 staining made it possible to differentiate $CD25^{hi}CD127^{lo}$ Treg from $CD25^{+}CD127^{+}$ activated Teff, which was not possible in the experimental setup used at 21 and 30 months.

After incubation with GAD_{65} for 7 days, we found increased frequencies of both $CD25^{hi}CD127^{lo}$ and $CD25^{+}CD127^{+}$ cells in GAD-alum treated patients (Fig 15). The increase in $CD25^{+}CD127^{+}$ cell frequency was more pronounced than the increase in $CD25^{hi}CD127^{lo}$ frequency. Thus, although there was a GAD_{65}-induced increase of Treg, stimulation with GAD_{65} also gave rise to a population of activated Teff in GAD-alum treated individuals. In our previous study, GAD_{65} stimulation had no effect on the frequency of $CD4^{+}CD25^{+}$ cells. The incongruous results could be due to different incubation times, as cells were incubated for 18 hours in the previous study compared to 7 days in the 4-year follow-up. The increase in Treg frequencies was comparable to those observed in the 2D group in the phase III trial, while the Teff frequencies were slightly lower which is not surprising considering the considerably longer duration after treatment in the 4-year follow-up. Since there were no major differences in effects on Treg and Teff frequencies, these immunological parameters cannot explain the differences in clinical outcomes observed in the two trials.

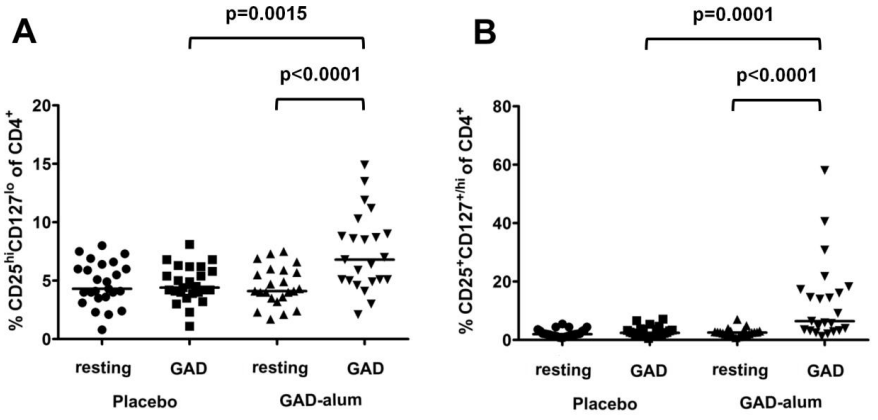

***Figure 15. A**. Frequency of $CD4^+CD25^{hi}CD127^{lo}$ cells (Treg) among resting and GAD_{65}-stimulated cells from placebo and GAD-alum treated patients. **B**. Frequency of $CD4^+CD25^+CD127^{+/hi}$ cells (Teff).*

Both non-suppressive and suppressive Treg are induced by antigen recall

Based on findings of Miyara *et al*, $FOXP3^+$ cells were divided into subsets of activated $FOXP3^{hi}CD45RA^-$ Treg, non-suppressive $FOXP3^{lo}CD45RA^-$ cells and $FOXP3^{lo}CD45RA^+$ resting Treg [118], at 9, 15 and 21 months. Miyaras findings did not come to our attention before initiation of the trial, hence why this analysis was not performed from baseline.

GAD_{65}-stimulation increased the frequency of mainly non-suppressive $FOXP3^{lo}CD45RA^-$ cells at 9, 15 and 21 months after treatment. This further supports our previous conclusion that a significant proportion of $FOXP3^+$ cells are activated Teff rather than suppressive Treg (Fig 16A). We proceeded to analyze expression patterns of HLA-DR and CD45RA on $CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ Treg based on findings reported by Schaier *et al* [265]. We found an increased frequency of $CD45RA^-HLA\text{-}DR^{hi}$ and $CD45RA^-HLA\text{-}DR^{dim}$ cells concomitant to a decreased frequency of $CD45RA^-HLA\text{-}DR^-$ and $CD45RA^+HLA\text{-}DR^-$ cells after GAD_{65}-stimulation at all follow ups at which this analysis was performed (9, 15 and 21 months)(Fig 16B). $HLA\text{-}DR^+$ Treg and $CD45RA^-HLA\text{-}DR^{hi}$ Treg in particular have been shown to be more potent suppressors of responder T cells [123-124]. Taken together these results indicate that while a large proportion of induced $FOXP3^+$ cells do not have the phenotype of suppressive Treg, a highly suppressive subset of Treg might still be induced by antigen recall after GAD-alum treatment.

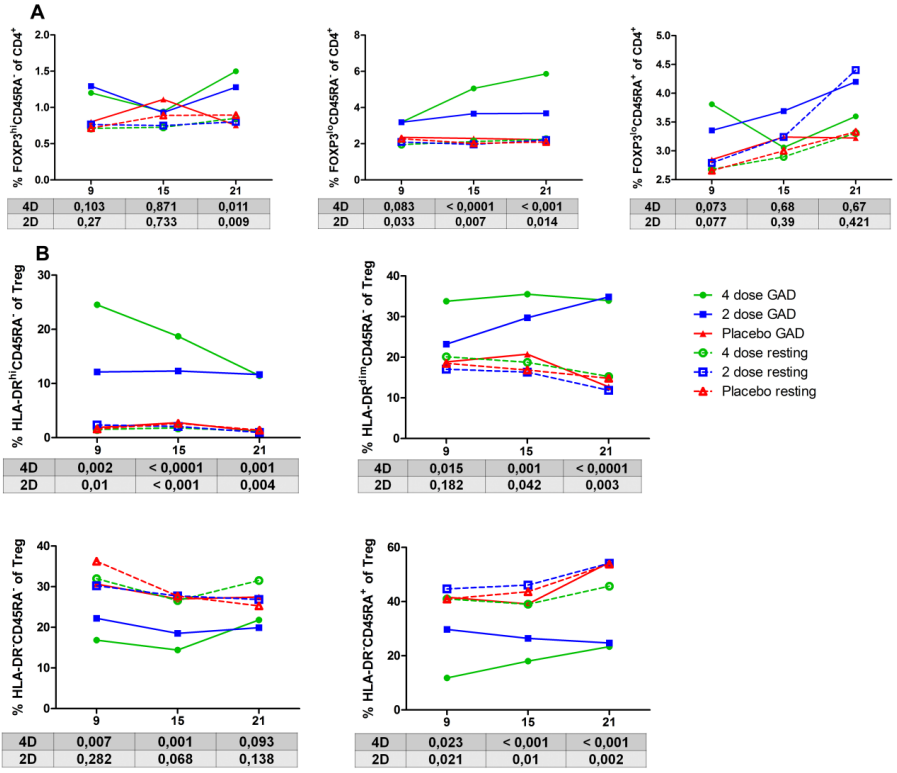

Figure 16. A*. Frequencies of FOXP3^{hi}CD45RA^{-} (activated Treg), FOXP3^{lo}CD45RA^{-} (non-suppressive) and FOXP3^{lo}CD45RA^{+} (resting Treg) are shown in left, center and right panel, respectively.* ***B****. Upper left panel shows frequencies of highly suppressive HLA-DR^{hi}CD45RA^{-} Treg in the CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+} Treg population. Upper right panel shows frequencies of HLA-DR^{dim}CD45RA^{-} cells, also reported to be more suppressive. Lower panels show frequencies of HLA-DR^{-}CD45RA^{-} and CD45RA^{+} cells. P-values indicate comparisons of GAD$_{65}$ stimulated cells from 4D and 2D groups compared to placebo.*

Antigen recall induces activated and memory T cells

The expression of CD45RO on CD4^{+} cells was also increased by GAD$_{65}$-stimulation in both 2D and 4D groups compared to placebo at 3, 9, 15 and 21 months, indicating induction of memory T cells in response to antigen recall (Fig 17A). A population of FSC^{hi}SSC^{hi} cells appeared at 3 months in GAD-alum treated patients upon GAD$_{65}$-stimulation, confirming our findings from the 4-year follow-up of the phase II study (Fig 17B). The FSC^{hi}SSC^{hi} population consisted mainly of CD25^{+}CD127^{+} and CD45RO^{+} cells (Fig 17C), and had high expression of FOXP3, CTLA-4 and HLA-DR. Furthermore the frequency of non-suppressive

FOXP3^{lo}CD45RA^{-} cells was increased in this population while the frequency of FOXP3^{lo}CD45RA^{+} resting Treg was decreased (Fig 17D). These results are all consistent with induction of activated T cells in response to antigen recall.

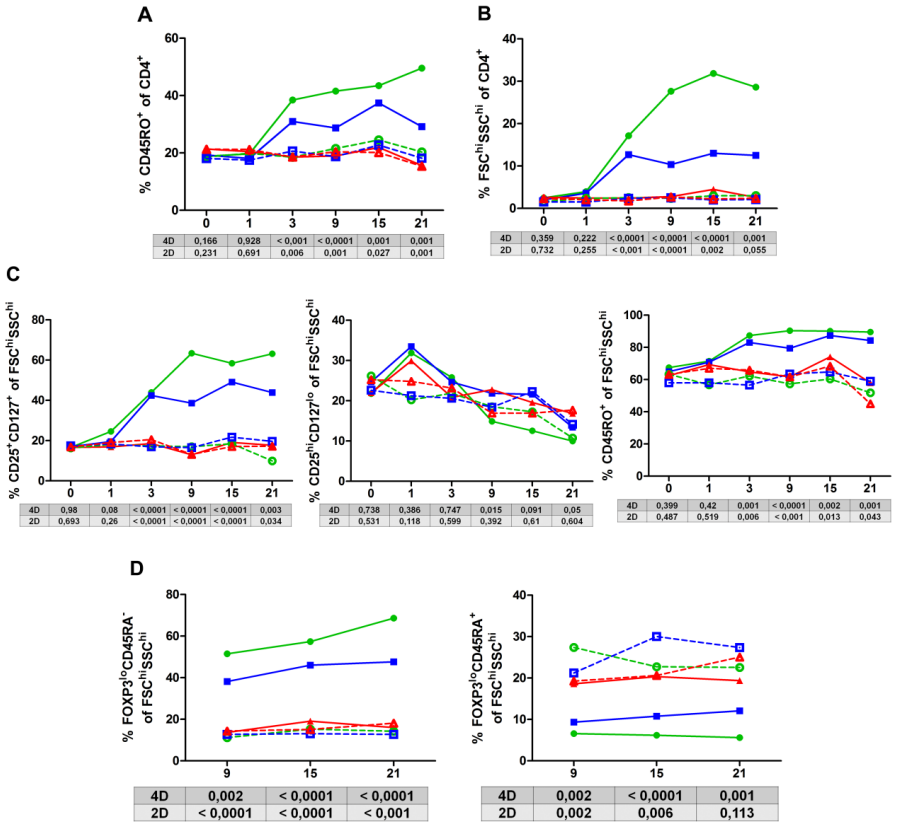

***Figure 17. A**. Frequencies of CD45RO^{+} memory T cells. **B**. Frequencies of FSC^{hi}SSC^{hi} cells. **C**. Frequencies of CD25^{+}CD127^{+} (Teff), CD25^{hi}CD127^{lo} (Treg) and CD45RO^{+} memory cells in the FSC^{hi}SSC^{hi} population. **D**. Frequencies of non-suppressive FOXP3^{lo}CD45RA^{-} and resting FOXP3^{lo}CD45RA^{+} Treg in the FSC^{hi}SSC^{hi} population. P-values indicate comparisons of GAD$_{65}$ stimulated cells from 4D and 2D groups compared to placebo.*

Antigen recall induces activated T cells 4 years after GAD-alum treatment

Stimulation with GAD_{65} induced a population with higher FSC and SSC than resting lymphocytes in 16 of 24 GAD-alum treated patients (Fig 18 A-B), as we also reported in a prior publication in which this population was found to consist mainly of $CD4^{+}CD45RO^{+}$ memory T cells [246]. In paper II we could further show that the $CD4^{+}FSC^{hi}SSC^{hi}$ population had high proportions of $CD25^{hi}CD127^{lo}$ (25%), $CD25^{+}CD127^{+}$ (46%) and $FOXP3^{+}$ cells (74%), and a higher expression of CD4 (Fig 18C-D). This is consistent with both a Treg and an activated Teff phenotype, and makes it difficult to draw a clear-cut conclusion about the composition of this population. However, the higher FSC and SSC by itself indicates that these cells are activated. Additionally, $FSC^{hi}SSC^{hi}$ cells exhibited higher CD4 MFI, and a cut-off using increased CD4 MFI between resting and GAD_{65}-stimulated cells was used to determine the number of patients in which a significant $FSC^{hi}SSC^{hi}$ population was induced by antigen recall (Fig 18D). The emergence of the $FSC^{hi}SSC^{hi}$ population was observed in both trials, and its composition was similar in both trials with high frequencies of $CD25^{+}CD127^{+}$, $CD45RO^{+}$ and $FOXP3^{+}$ cells. Thus, differences in antigen recall induced $FSC^{hi}SSC^{hi}$ cells do not reflect differences in clinical outcomes between the trials.

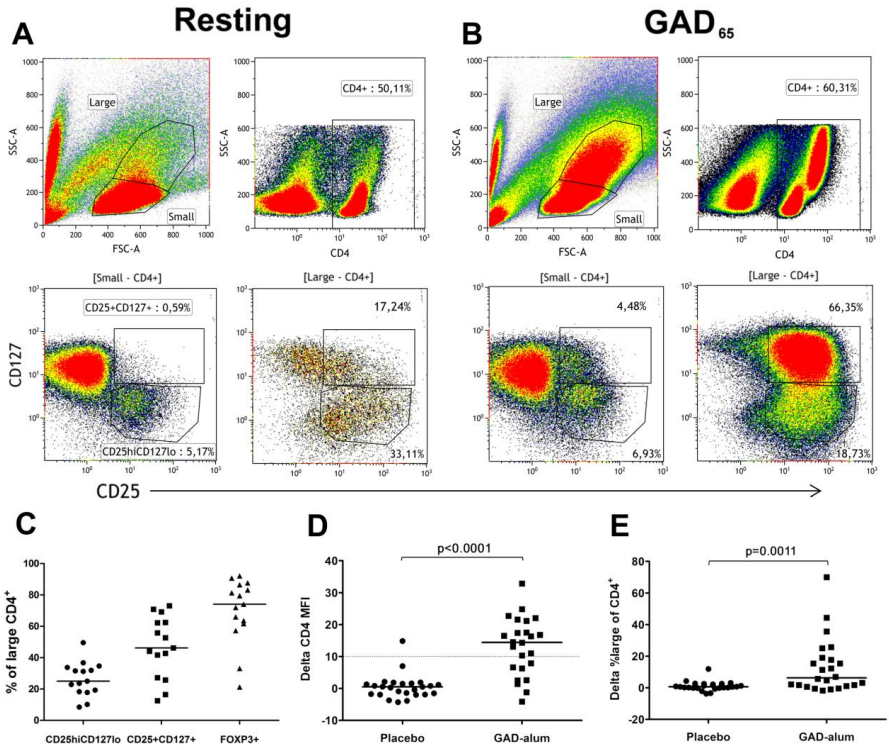

***Figure 18. A**. Illustration of gates used to discriminate $FSC^{hi}SSC^{hi}$ (large) lymphocytes from normal (small), and representative gating of $CD25^{hi}CD127^{lo}$ (Treg) and $CD25^{+}CD127^{+}$ (Teff) cells from normal and $FSC^{hi}SSC^{hi}$ cells in resting cells. **B**. Illustration of the same gates in cells stimulated with GAD_{65}, clearly demonstrating the emergence of a significant $FSC^{hi}SSC^{hi}$ population with higher CD4 expression and strong induction of $CD25^{+}CD127^{+}$ cells in a representative experiment. **C**. Frequencies of $CD25^{hi}CD127^{lo}$, $CD25^{+}CD127^{+}$ and $FOXP3^{+}$ cells in the $CD4^{+}FSC^{hi}SSC^{hi}$ population. **D**. Antigen recall induced intensity of CD4 expression in placebo and GAD-alum treated patients, dashed line indicates cut-off classifying patients as $FSC^{hi}SSC^{hi}$ positive. **E**. Antigen recall induced change in frequency of $FSC^{hi}SSC^{hi}$ cells.*

FOXP3 expression in the $CD4^+$ and the $CD4^+FSC^{hi}SSC^{hi}$ population was significantly increased after incubation with GAD_{65} in GAD-alum treated patients (Fig 19). The proportion of $CD4^+CD25^{hi}CD127^{lo}$ cells co-expressing FOXP3 and CD39 was also significantly higher in GAD-alum treated patients following GAD_{65} stimulation. In summary, our conclusion is that *in vitro* GAD-recall leads to expansion of both Treg and activated Teff populations.

CD101 had been suggested to be preferentially expressed on Treg in mice and to be associated with higher suppressive function [266]. We found the expression of CD101 to be extremely heterogenous on human T cells, and its expression was similar on conventional T cells and Treg. We therefore excluded it from our analysis. Subsequent studies in humans have not confirmed the higher suppressive capacity of $CD101^+$ Treg observed in mice [267].

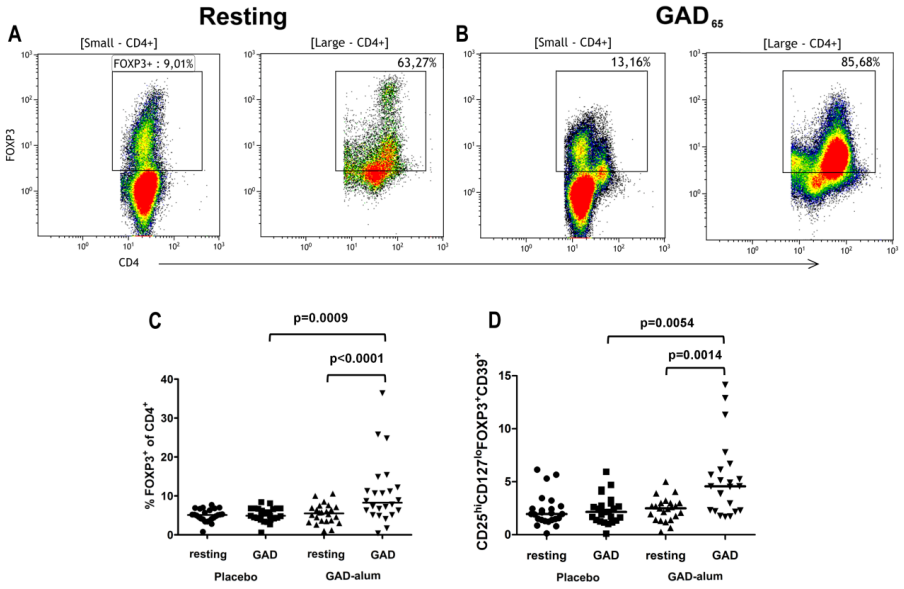

Figure 19. A-B*. Gating of $FOXP3^+$ cells in normal and $FSC^{hi}SSC^{hi}$ cells at resting conditions and stimulated with GAD_{65}, respectively.* ***C****. Frequency of $FOXP3^+$ cells.* ***D****. Frequency of $CD25^{hi}CD127^{lo}$ cells co-expressing FOXP3 and CD39.*

GAD$_{65}$-specific T cells are of a memory and activated phenotype

The frequency of GAD$_{65}$-specific CD4$^+$ T cells was analyzed using DR3- and DR4-restricted HLA class II tetramers loaded with GAD$_{65}$ peptides. Data from 1-21 months was pooled due to low number of performed experiments. GAD$_{65}$-stimulation induced higher frequencies of CD4$^+$TMR$^+$ cells in both treated groups (Fig 20A). TMR$^+$ cells consisted mainly of CD25$^+$CD127$^+$ cells after GAD$_{65}$-stimulation in treated patients, while the frequency of CD25^{hi}CD127^{lo} was the same as in placebo treated patients (Fig 20C-D). The expression of CD45RO was also increased on TMR$^+$ cells after GAD$_{65}$-stimulation in treated patients (Fig 20E). While the frequency of TMR$^+$ cells in the FSC^{hi}SSC^{hi} population was unchanged after GAD-stimulation, the FSC^{hi}SSC^{hi} population contained a higher proportion of TMR$^+$ cells than the total CD4$^+$ population regardless of stimuli (Fig 20F). We thus concluded that the GAD$_{65}$-specific population mainly consisted of cells with a memory or activated phenotype after antigen recall.

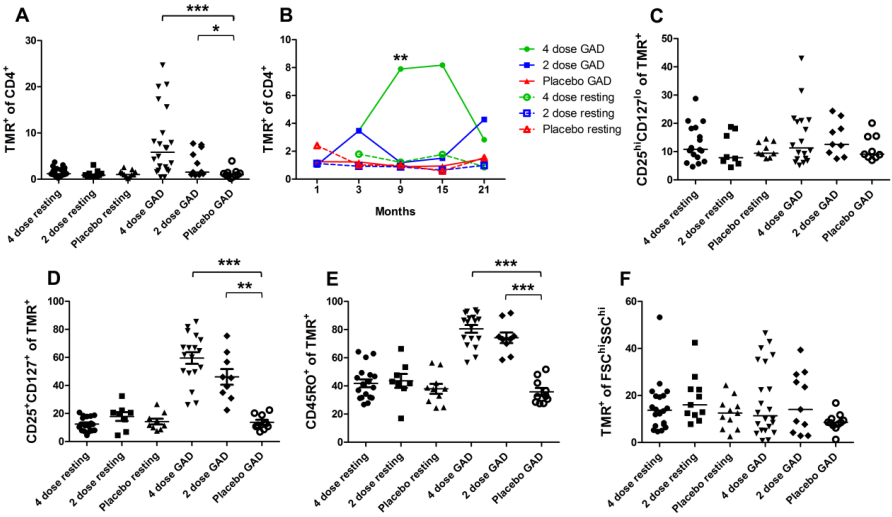

***Figure 20. A**. Frequencies of GAD$_{65}$-specific CD4$^+$TMR$^+$ cells in pooled experiments from 1-21 months. **B**. TMR$^+$ cell frequency over the course of the trial. **C**. Frequency of CD25^{hi}CD127^{lo} (Treg) in the GAD$_{65}$-specific TMR$^+$ population. **D**. Frequency of CD25$^+$CD127$^+$ (Teff) cells in the TMR$^+$ population. **E**. Frequency of TMR$^+$CD45RO$^+$ GAD$_{65}$-specific memory T cells. **F**. Frequency of TMR$^+$ cells in the FSC^{hi}SSC^{hi} population. * = p<0.05, ** = p<0.01, *** = p<0.001.*

Activated Teff proliferate in response to antigen recall

Finally, proliferation of PBMC after incubation with GAD_{65} was measured by CFSE dilution at baseline, 1, 3 and 9 months, in conjunction with markers of T cell. Our group performed a general proliferation assay using PBMC based on ^{3}H-Thymidine incorporation in a larger subset of patients which showed antigen specific proliferation in response to GAD_{65} in treated individuals. The flow cytometric analysis of proliferation was employed to elucidate which specific subsets of lymphocytes proliferated in response to antigen recall, since this cannot be revealed by a thymidine incorporation assay using total PBMC. The proliferation of $CD4^{+}$, $CD8^{+}$, $CD25^{+}CD127^{+}$ and $CD25^{hi}CD127^{lo}$ cells was analyzed. A proliferative response was evident at 3 and 9 months but not at 1 month after treatment, thus data from 3 and 9 months was pooled for statistical analysis. Both $CD4^{+}$ and $CD8^{+}$ T cells were found to proliferate on antigen recall in treated patients, but the frequency of proliferating $CD4^{+}$ cells was higher than proliferating $CD8^{+}$ cells (Fig 21A-B). Proliferating cells were most commonly found in the $FSC^{hi}SSC^{hi}$ population (Fig 21C). The proportion of $CD4^{+}CD25^{+}CD127^{+}$ cells among dividing cells was significantly increased in treated patients compared to placebo whereas the frequency of dividing $CD4^{+}CD25^{hi}CD127^{lo}$ cells was not different between groups (Fig 21D-E). Resting experiments are not shown due to the low number of proliferating cells under resting conditions, which makes frequency data unreliable. While we were unable to analyze CD45RO expression in our proliferation panel, given our separate findings that $FSC^{hi}SSC^{hi}$ cells were commonly $CD45RO^{+}$ and were also commonly proliferating in our CFSE dilution assay, it could be extrapolated that a significant proportion of proliferating cells are $CD45RO^{+}$. Since this antigen specific stimulation in the presence of APC *in vitro* results in proliferation of activated T cells in GAD-alum treated patients, but not in placebo treated patients, it could be speculated that the treatment does not induce Treg capable of suppressing an immune response directed toward GAD_{65}.

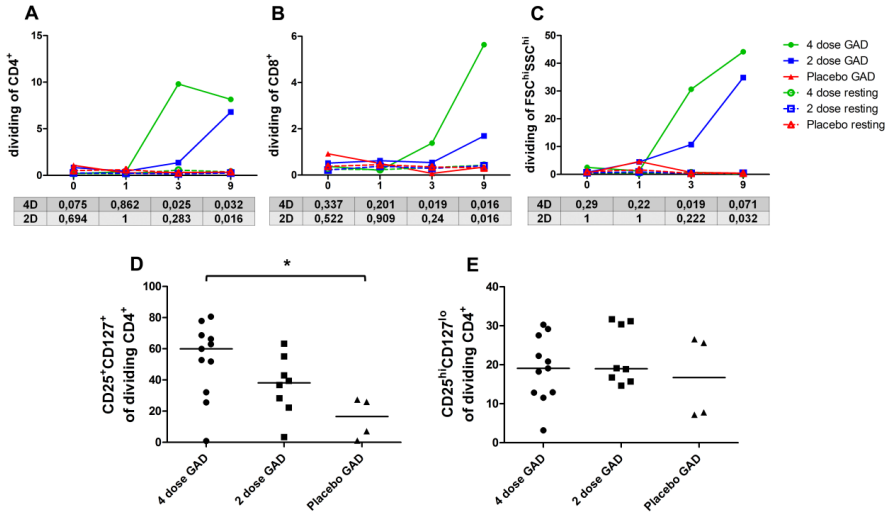

***Figure 21. A**. Frequencies of proliferating cells in the CD4$^+$ population. **B**. Frequencies of proliferating cells in the CD8$^+$ population. **C**. Frequencies of FSC^{hi}SSC^{hi} cells that were proliferating. **D**. Freqeuncy of CD25$^+$CD127$^+$ (Teff) cells among proliferating cells, using pooled data from 3 and 9 months. **E**. Frequency of CD25^{hi}CD127^{lo} (Treg) cells in the proliferating population.*

The assay we employed is very much focused on antigen recall induced effects on T cell populations, since we were mainly interested in GAD_{65}-specific immune regulation. The resting cells that were incubated for 7 days without stimulation as controls are perhaps unlikely to retain their *ex vivo* phenotype, and might not represent the frequencies of T cell population in the periphery of the trial participants. It is thus possible that we may be unable to detect potential *in vivo* differences in T cell frequencies brought about by GAD-alum treatment due to experimental design in both GAD-alum trials.

Changes in T cell frequencies are not related to clinical outcome

In an effort to establish immunological markers of clinical efficacy, we tested whether expression of any marker that was affected by GAD_{65}-stimulation was related to clinical outcome in the 4-year follow-up of the phase II trial. We found no association between increased expression of any marker affected by antigen recall and changes in stimulated C-peptide measured as ΔAUC or AUC 4 years after treatment. Neither was C-peptide secretion affected in patients where an FSC^{hi}SSC^{hi} population was induced by antigen recall.

The lack of clinical effect in the phase III trial precluded this analysis in Paper III.

Effects of additional doses of GAD-alum

The GAD-alum treated groups receiving either two or four doses of GAD-alum in the phase III trial were treated identically until the 3-month follow-up when the 4D group received their third injection which means any effects of additional doses should be detectable 9 months after start of treatment. Thus, we determined whether additional doses of GAD-alum had any effect on T cell populations 9, 15 and 21 months after start of treatment. The 4D group had significantly higher frequencies of $CD4^+CD25^+CD127^+$ and $CD4^+CD45RO^+$ cells compared to the 2D group at 9 and 21 months. The frequency of $FSC^{hi}SSC^{hi}$ cells was also significantly higher in the 4D group at 9 and 21 months, while the 4D group exhibited significantly stronger proliferation in response to antigen recall at 9 and 15 months, as well as higher frequencies of $CD4^+FOXP3^{lo}CD45RA^-$ cells at 15 and 21 months. Additional doses of GAD-alum in the 4D group did not affect the frequency of $CD25^{hi}CD127^{lo}$ cells or the expression of FOXP3, CTLA-4 or HLA-DR on total $CD4^+$ cells, but their expression in the $CD25^+CD127^+$ population was significantly higher in the 4D group after antigen recall at 9, 15 and 21 months. Administration of additional doses had no effect on the frequency of GAD_{65}-specific cells or the composition of the TMR^+ population, nor did it affect the phenotype of proliferating cells. These results indicate that additional doses of GAD-alum amplifies the antigen recall induced expansion of memory, activated and $CD4^+FOXP3^{lo}CD45RA^-$ T cells, but does not affect the frequency of Treg. From an immunological perspective it would thus seem that additional doses of GAD-alum are not beneficial in inducing tolerance to GAD_{65}. In this regard it is interesting that our group observed a significant preservation of β-cell function in the 2D group compared to placebo in a subgroup of Swedish patients that completed the 30 month follow-up of the phase III trial before it was closed, but not in the 4D group.

Induced expression of T cell markers correlates with cytokine secretion

We performed correlation analyses to determine whether GAD_{65}-induced changes in phenotypical markers or morphology coincided with GAD_{65}-induced secretion of IFN-γ, IL-10, IL-13, IL-17, IL-5 and TNF as previously determined by Luminex by our group [268]. We found that the frequencies of $FSC^{hi}SSC^{hi}$, $CTLA\text{-}4^+$, $HLA\text{-}DR^+$, $CD45RO^+$, $CD25^+CD127^+$ and $CD25^{hi}CD127^{lo}$ cells correlated significantly with secretion of all the above mentioned cytokines 3, 9, 15 and 21 months after treatment, with few exceptions (Table VI).

This indicates that there is no distinct correlation between a specific population of T cells and production of specific cytokines, but rather it supports our previous findings suggesting that GAD-alum treatment has an immunomodulatory effect leading to an antigen recall induced across-the-board immune response in the majority of patients. It is clear, however, that the patients in which antigen recall results in changes in frequencies of T cell populations are the same that respond with cytokine secretion in a separate assay. The correlation between cytokine secretion and frequencies of $FSC^{hi}SSC^{hi}$ cells as well as $CD25^{+}CD127^{+}$ cells were generally strongest, i.e. achieved the highest correlation coefficients, indicating that the frequency of these cells is slightly more robustly associated with cytokine secretion.

Table VI. Correlation of cytokine secretion and T cell frequencies.

Population	Cytokine	9 Months	15 Months	21 Months
$FSC^{hi}SSC^{hi}$		r / p	r / p	r / p
	IFN-γ	0.71 / <0.001	0.69 / <0.001	0.38 / 0.011
	IL-10	0.67 / <0.001	0.72 / <0.001	0.41 / 0.005
	IL-13	0.75 / <0.001	0.71 / <0.001	0.53 / <0.001
	IL-17	0.65 / <0.001	0.27 / 0.06	0.26 / 0.08
	IL-5	0.79 / <0.001	0.80 / <0.001	0.69 / <0.001
	TNF	0.59 / <0.001	0.58 / <0.001	0.42 / 0.004
$CD25^{+}CD127^{+}$		r / p	r / p	r / p
	IFN-γ	0.72 / <0.001	0.66 / <0.001	0.14 / 0.54
	IL-10	0.66 / <0.001	0.68 / <0.001	0.23 / 0.30
	IL-13	0.76 / <0.001	0.68 / <0.001	0.56 / 0.007
	IL-17	0.63 / <0.001	0.23 / 0.11	0.57 / 0.005
	IL-5	0.80 / <0.001	0.80 / <0.001	0.67 / <0.001
	TNF	0.60 / <0.001	0.58 / <0.001	0.28 / 0.08
$CTLA\text{-}4^{+}$		r / p	r / p	r / p
	IFN-γ	0.36 / 0.001	0.62 / <0.001	0.42 / 0.004
	IL-10	0.39 / <0.001	0.46 / <0.001	0.53 / <0.001
	IL-13	0.40 / <0.001	0.47 / <0.001	0.57 / <0.001
	IL-17	0.33 / 0.004	0.35 / 0.002	0.44 / 0.002
	IL-5	0.37 / 0.001	0.5 / <0.001	0.55 / <0.001
	TNF	0.3 / 0.01	0.49 / <0.001	0.35 / 0.015
$FOXP3^{+}$		r / p	r / p	r / p
	IFN-γ	0.34 / 0.003	0.002 / 0.98	0.65 / <0.001
	IL-10	0.29 / 0.011	0.08 / 0.51	0.57 / <0.001
	IL-13	0.38 / <0.001	0.07 / 0.52	0.63 / <0.001
	IL-17	0.28 / 0.016	0.04 / 0.74	0.39 / 0.006
	IL-5	0.33 / 0.004	0.11 / 0.33	0.62 / <0.001
	TNF	0.31 / 0.006	0.15 / 0.19	0.56 / <0.001

Correlation of frequencies of selected T-cell populations and GAD_{65}-induced secretion of cytokines determined by Luminex at 9, 15 and 21 months in the phase III trial. r = Spearman's rank correlation coefficient. Correlations were determined in the entire trial population, i.e. placebo, 2D and 4D combined.

Treg function in GAD-alum treatment and T1D

We demonstrated increased frequency of both Treg and Teff upon antigen recall after treatment with GAD-alum, but these changes were not related to clinical outcome 4 years after treatment. This, in conjunction with accumulating data suggesting Treg-mediated suppression is impaired in patients with T1D, fueled our interest in elucidating whether GAD-alum treatment had any effect on the suppressive capacity of Treg.

Treg function is not affected by GAD-alum treatment

To clarify whether Treg play a mechanistic role in the clinical effect of GAD-alum treatment we performed suppression assays using Treg and Teff expanded from participants in the GAD-alum phase II trial.

We found that Treg from patients treated with GAD-alum suppressed the proliferation of polyclonally stimulated autologous Teff to the same extent as Treg from patients receiving placebo (Fig 22A). It would have been valuable to compare clinical parameters to Treg suppressive function, but the small number of patients included in the suppression assay and the fact that no patients classified as responders to treatment were randomly included in the assay precluded such analysis.

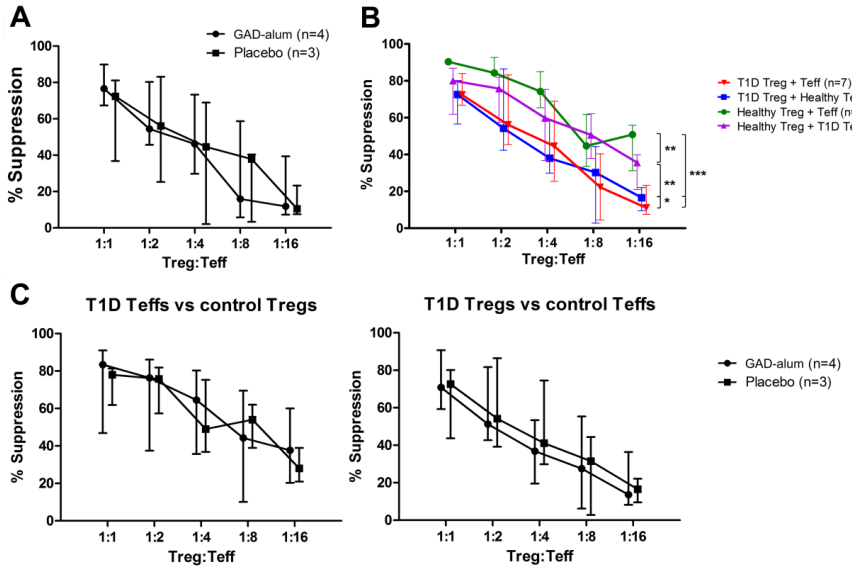

Figure 22. A*. Treg-mediated suppression of proliferation in autologous co-cultures of Treg and Teff from GAD-alum (●) and placebo treated (■) patients at decreasing Treg:Teff ratios.* ***B****. Suppression exerted by Treg from patients with T1D in co-culture with autologous Teff (red downward triangle) and Teff from a healthy control (blue square), and suppression exerted by Treg from a healthy control in culture with autologous Teff (green circle) and Teff from patients with T1D (purple upward triangle).* ***C****. Left panel shows Treg mediated suppression in co-cultures of T1D Teff and healthy Treg, right panel shows suppression exerted by T1D Treg in co-culture with healthy Teff, using cells from GAD-alum (●) and placebo treated (■) patients.*

Treg-mediated suppression is impaired in patients with T1D

Treg-mediated suppression in patients with T1D was compared to suppression exerted by cells from a healthy individual. Treg from patients with T1D suppressed autologous Teff less efficiently than Treg from the healthy control in culture with autologous Teff (Fig 22B). This is in accordance with previously published data from other groups [163,169]. Both Schneider *et al* and Lawson *et al* went on to test whether impaired suppression was intrinsic to the Treg or Teff population and both groups found that Teff from patients with T1D were resistant to Treg mediated suppression even if the Treg originated from healthy controls, rather than Treg from patients with T1D exerting less efficient suppression. We performed the same experiment, testing Treg from patients with T1D against Teff from a healthy control and vice versa. Our results contradicted the findings of Schneider *et al* and Lawson *et al*, instead indicating that Treg from patients with T1D were able to suppress neither autologous Teff nor Teff from the healthy control as potently as healthy Treg (Fig 22B). We further found that this affected both GAD-alum and placebo treated patients equally (Fig 22C).

Our finding that Treg-mediated suppression is impaired in patients with T1D has implications for our previous finding that patients with recent onset T1D had higher frequencies of Treg-like cells. It could be speculated that the immune system attempts to halt the autoimmune process by producing larger numbers of Treg in T1D and pre-diabetes, but since the Treg of patients with T1D are deficient in their suppressive function, an increase in their number may be inadequate in counteracting T1D. It remains to be elucidated how the suppressive function of Treg in patients with T1D breaks down. As discussed, CTLA-4 and CD39 are both involved in Treg suppressive function, but we have demonstrated high expression of both markers in patients with T1D which make them unlikely players in this regard. The phenomenon may well be highly heterogenous in nature since other groups have reported that impaired suppression is dependent on resistant Teff. Further studies are clearly needed to address these issues.

We could demonstrate that the expanded cells used in the proliferation assay were lineage specific after expansion, which is important to ensure validity of results from the suppression assays. Treg maintained high expression of FOXP3 and CTLA-4, and were still $CD25^{hi}CD127^{lo}$ after the expansion process. Meanwhile, the frequency of $CD25^{hi}CD127^{lo}$, $FOXP3^{+}$ and $CTLA\text{-}4^{+}$ cells among expanded Teff was low, approximately 5%, 10% and 15% respectively. Sorted cells of both lineages also expanded to similar degrees in terms of fold change in cell number regardless if they originated in patients treated with GAD-alum or

placebo. Gene expression patterns were also analyzed in expanded cells, and Treg had higher expression of CD25 and FOXP3 mRNA, but lower expression of IL-2 compared to Teff, which is also consistent with lineage specificity. We also analyzed cytokine secretion in supernatants from proliferation assays of Treg and Teff and found that Teff, but not Treg, secreted IFN-γ, IL-13, IL-5 and TNF. Supernatants from stimulated Teff also contained higher concentrations of IL-2 than Treg supernatants, while the secretion of IL-10 was similar in both subsets. Secretion of IFN-γ, IL-13 and IL-5 was furthermore suppressed in co-cultures in a reasonably dose-dependent manner, with no difference between cells from GAD-alum and placebo treated patients.

One should consider the fact that the suppression assay we employed was not antigen specific but relied on monoclonal antibodies against T cell receptors to induce T cell activation and proliferation. First, the polyclonal nature of stimulation may mask antigen specific effects on suppression in patients with T1D, and in patients treated with GAD-alum in particular. Antigen specific suppression assays are much more technically demanding and we were not able to perform such experiments in the phase II trial, but our group plans to proceed with these experiments in the future using cells expanded from patients participating in the phase III trial. Secondly, the assay does not include APC such as DC, and considering that several mechanisms of action involved in Treg-mediated suppression may affect DC and their ability to present antigen and co-stimulation to Teff as discussed previously, the assay may not properly reflect *in vivo* Treg-mediated suppression. Furthermore it has been argued that thymidine incorporation is unsuitable to measure Treg-mediated suppression of proliferation in co-culture assays because $CD45RA^+$ Treg proliferate *in vitro* [125]. However, our assay includes wells containing purified Treg exclusively, which did not proliferate significantly in response to polyclonal stimulation.

Effects of NALP3 inflammasome polymorphisms on T1D risk and severity

We sought to determine whether NALP3 inflammasome gene polymorphisms had any effect on risk of developing T1D, disease severity, or efficacy of GAD-alum treatment since alum is known to rely on the inflammasome to exert adjuvanticity. To this end, we genotyped three SNPs in genes encoding components of the inflammasome; the Q705K SNP in the NLRP3 gene which is a moderate gain-of-function mutation, CARD8 C10X and an SNP downstream of the NLRP3 gene, rs10733113. The minor allele of CARD8 C10X introduces a premature

stop codon that produces a truncated protein, resulting in decreased nuclear factor κB inhibition, while the SNP rs10733113 is located in a regulatory region and is associated with decreased NALP3 expression and impaired IL-1β production. Genetic analysis included 181 patients with T1D, 63 participants in the GAD-alum phase II trial and 793 control individuals selected from a population registry matching the hospital recruitment area. To assess effects of polymorphisms on T1D progression, we used positivity for autoantibodies as well as fasting and stimulated C-peptide levels as markers of disease severity. C-peptide data was available for 87 patients, whereas autoantibody titers were available for 73 patients with T1D. SNP genotype was further related to clinical parameters for participants in the GAD-alum phase II trial. A breakdown of available data for patients with T1D and participants in the GAD-alum phase II trial is given in Figure 23.

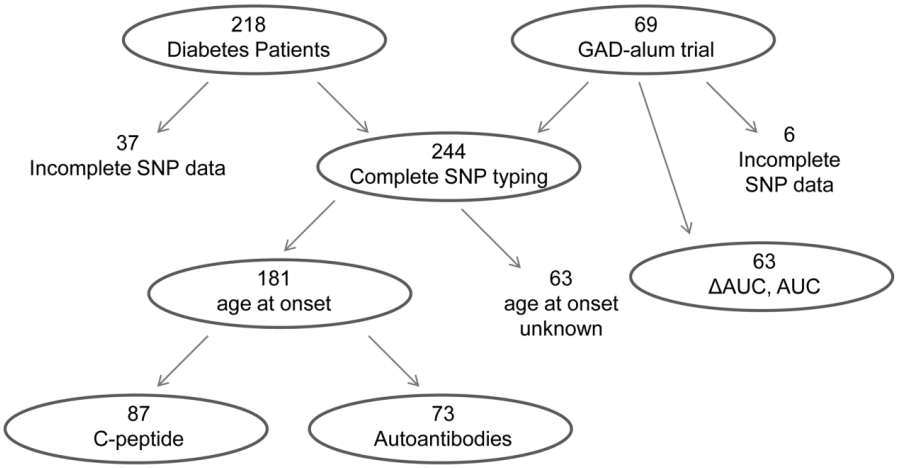

Figure 23. *Illustration of data availability for analyses of SNP effects on aspects of T1D performed in Paper IV. Genetic analysis for T1D (n= 181), GAD-alum patients (n=63) and control individuals (n=793). Effect of polymorphisms on T1D progression was related to C-peptide levels (n= 87 patients) and autoantibody titers (n= 73) in the T1D patients. SNP genotype was related to clinical parameters for participants in the GAD-alum phase II trial (n=63).*

NLRP3 and CARD8 polymorphisms do not affect risk of T1D

We found no difference in the distribution of alleles between patients with T1D, which also included patients participating in the GAD-alum trial, and the control population. All three typed SNPs were in Hardy-Weinberg equilibrium. The distribution of alleles is given in Table VII. We proceeded to test males and females separately since different allele distributions have been demonstrated previously in male patients with Crohn's disease [253]. We found no difference in allele distribution in either sex, nor could we demonstrate any effect of combinations of several polymorphisms on T1D risk. We also analyzed risk of T1D in homozygotes and heterozygotes for variant alleles compared to homozygous wild type individuals and found no significant effect on risk, as described in Table VIII. Despite a strong rationale for an involvement of the inflammasome in the pathogenesis of T1D, we could find no evidence for SNPs in genes encoding inflammasome components as predisposing factors for developing T1D.

Table VII. Distribution of alleles.

SNP	Alleles	T1D n=244	Control n=793
NLRP3 Q705K			
	CC	215 (88.1)	693 (87.4)
	CA	29 (11.9)	94 (11.9)
	AA	0 (0)	6 (0.7)
CARD8 C10X			
	TT	109 (44.7)	331 (41.7)
	AT	104 (42.6)	359 (45.3)
	CA	31 (12.7)	103 (13)
rs10733113			
	AA	177 (72.5)	575 (72.5)
	AG	64 (26.2)	204 (25.7)
	GG	3 (1.2)	14 (1.8)

Distribution of alleles is given as number of individuals with the given alleles, and percentage of population with the given alleles in parentheses.

Table VIII. Genotype Frequencies in male and female patients and controls.

Sex / Genotype	T1D	Control	OR (95% CI)	P
Males				
rs10733113 AA	57 (70.4)	287 (72.6)	1	
rs10733113 AG/GG	24 (29.6)	108 (27.3)	1.12 (0.66-1.89)	1
Females				
rs10733113 AA	70 (70)	287 (72.3)	1	
rs10733113 AG/GG	30 (30)	110 (27.7)	1.12 (0.69-1.81)	1
Males				
NLRP3 Q705K CC	71 (87.6)	355 (89.9)	1	
NLRP3 Q705K CA/AA	10 (12.4)	40 (10.1)	1.25 (0.59-2.61)	1
Females				
NLRP3 Q705K CC	91 (91)	337 (84.9)	1	
NLRP3 Q705K CA/AA	9 (9)	110 (15.1)	0.55 (0.26-1.16)	0.35
Males				
CARD8 C10X TT	32 (39.5)	174 (44)	1	
CARD8 C10X TA/AA	49 (60.5)	221 (56)	1.2 (0.74-1.96)	1
Females				
CARD8 C10X TT	51 (51)	156 (39.3)	1	
CARD8 C10X TA/AA	49 (49)	241 (60.7)	0.62 (0.4-0.96)	0.11
Males				
Q705K/C10X CC TT	26 (32.1)	152 (38.5)	1	
Q705K/C10X CA/AA TT	6 (7.4)	22 (5.5)	1.59 (0.59-4.31)	1
Q705K/C10X CC TA/AA	45 (55.5)	203 (51.4)	1.29 (0.76-2.19)	1
Q705K/C10X CA/AA TA/AA	4 (5)	18 (4.6)	1.3 (0.41-4.14)	1
Females				
Q705K/C10X CC TT	45 (45)	129 (32.5)	1	
Q705K/C10X CA/AA TT	6 (6)	28 (7)	0.61 (0.23-1.57)	1
Q705K/C10X CC TA/AA	46 (46)	209 (52.5)	0.62 (0.39-0.99)	0.147
Q705K/C10X CA/AA TA/AA	3 (3)	32 (8)	0.26 (0.07-0.91)	0.105

Genotype frequencies are given as number of individuals with percentage of the population in parentheses. OR = Odds Ratio, CI = Confidence Interval. Odds Ratios are relative to wild type genotype.

Bonferroni correction was used in this study to compensate for multiple comparisons. It is perhaps debatable whether correction should be applied when only two or three different comparisons have been performed. Before applying Bonferroni correction, our data suggested a significantly decreased risk of T1D conferred by variant alleles at CARD8 C10X in females (OR=0.622), and a further decreased risk in females with variant alleles at both CARD8

C10X and NLRP3 Q705K (OR=0.266). These results were obtained when heterozygotes and homozygotes for variant alleles were grouped together as shown in Table VIII.

Effects of SNPs on T1D progression

Patients who are diagnosed with T1D at a younger age commonly have a worse prognosis. We compared age-at-onset of patients with variant alleles to patients with wild-type alleles and found no difference in how young patients were when they were diagnosed with T1D related to the tested SNPs. We further compared fasting C-peptide levels at diagnosis and stimulated C-peptide levels after a mixed meal tolerance test at 3 and 9 months after diagnosis, to assess the effect of variant alleles on insulin production. We could not find any differences in fasting C-peptide levels at diagnosis related to variant alleles. There was a trend toward decreased stimulated C-peptide secretion 3 months after diagnosis in males with one or two A alleles at CARD8 C10X, but the finding was not repeated at 9 months after diagnosis. Finally we examined whether variant alleles was related to the presence of autoantibodies against islet antigens, which could indicate an accelerated form of T1D. Patients with one or two G alleles at rs10733113 were more likely to produce two or three autoantibodies against insulin, GAD_{65} or IA-2 than patients with two A alleles, but there was no association with the NLRP3 Q705K or CARD8 C10X SNPs (Table IX). Unfortunately, none of the patients with T1D included in this study were homozygous for the variant allele at NLRP3 Q705K which is associated with a moderately hyperactive inflammasome. It might have been interesting to assess the effects of homozygous alleles at this SNP.

Table IX. Autoantibody positivity depending on alleles.

Genotype	**0-1 autoantibody n (%)**	**2-3 autoantibodies n (%)**
rs10733113 AA	21 (41.2)	30 (58.8)
rs10733113 AG/GG	3 (13.6)	19 (86.4)
NLRP3 Q705K CC	22 (34.9)	41 (65.1)
NLRP3 Q705K CA/AA	2 (20)	8 (80)
CARD8 C10X TT	11 (36.7)	19 (63.3)
CARD8 C10X TA/AA	13 (30.2)	30 (69.8)

Number of patients with wildtype and variant alleles who were positive for 0-1 and 2-3 autoantibodies against insulin, GAD_{65} and IA-2.

Effects of variant alleles on GAD-alum treatment

Since alum has been indicated as an inflammasome stimulus and inflammasome function is required for alum-adjuvanticity, one of our hypotheses was that GAD-alum treatment would be affected by polymorphic alleles. We compared stimulated C-peptide secretion measured by AUC and ΔAUC in patients participating in the GAD-alum trial. AUC tended to be higher among GAD-alum treated patients carrying at least one A allele at CARD8 C10X 21 months after the first of two injections of GAD-alum, compared to GAD-alum treated patients with the TT genotype. Furthermore, placebo treated patients with one or more G alleles at rs10733113 had less residual insulin secretion than AA placebo treated patients at 15 and 21 months, whereas only placebo treated patients with the G allele had significantly lower residual insulin secretion than AA GAD-alum treated patients at 9 and 30 months after treatment (Fig 24).

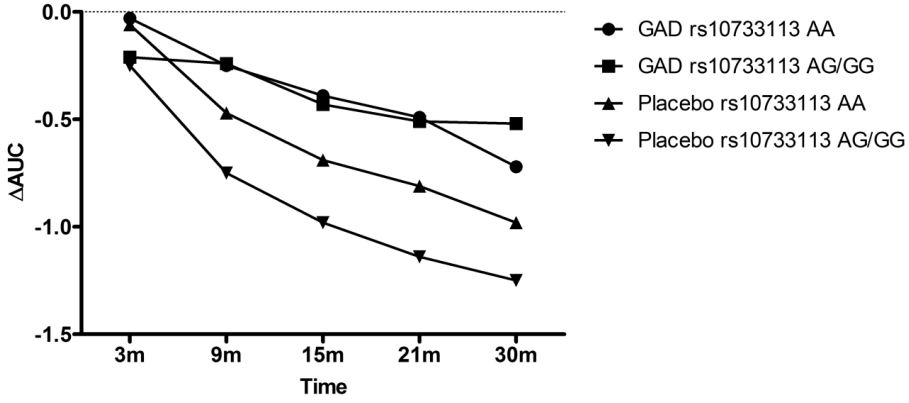

Figure 24. *Loss of C-peptide secretion over time in GAD-alum and placebo treated patients with wildtype alleles and heterozygous or homozygous variant alleles at the SNP rs10733113.*

At 15 months after treatment, placebo treated patients with the CC genotype of NLRP3 Q705K had lower ΔAUC than patients treated with GAD-alum with the same genotype (on average 0.38 nmol/l/2hr), whereas placebo treated patients carrying a Q705K A allele showed a further decrease in ΔAUC (on average 0.79 nmol/l/2hr). However, this effect was not noticeable at any other time point of the follow up. Patients treated with placebo who had the CARD8 C10X TA or AA genotypes had significantly lower ΔAUC than TT GAD-alum

treated patients at 30 months (p=0.039), whereas TT placebo treated patients did not differ significantly.

Our results indicate a small effect on insulin secretion in patients with variant alleles at CARD8 C10X, but this could only be observed at a single time point. Furthermore, GAD-alum treated patients seem to have higher residual insulin secretion than placebo treated patients with variant alleles at rs10733113, but not higher than wild-type placebo treated patients. A similar effect was seen sporadically in placebo treated patients with variant alleles at CARD C10X and NLRP3 Q705K. These findings suggest that the tested polymorphisms do not have a large effect on the efficacy of GAD-alum treatment, but did have an effect on disease progression in the placebo group in this trial. In particular, polymorphic alleles in the regulatory region of NLRP3 associated with decreased expression of NALP3 and hypoproduction of IL-1β seem to have an effect, but oddly, polymorphic alleles associated with an overactive inflammasome do not seem to have an effect. Polymorphic alleles producing a truncated CARD8 protein do not seem to have a profound effect on T1D risk or progression, but since it is uncertain whether CARD8 is a required component of the inflammasome, it is uncertain whether this affects inflammasome function or if its effect is mediated solely through decreased inhibition of nuclear factor κB signaling.

CONCLUDING REMARKS

Several attempts at preventing T1D or intervening in manifest T1D have been made during the last decades with variable success. Accumulating evidence for a role of Treg in the immunology of T1D and in the mechanism of action of GAD-alum treatment prompted our analysis of this important T cell subset in patients with T1D and participants in the GAD-alum clinical trials. Indications of important functions of the inflammasome and IL-1β in T1D pathogenesis and the Th2 polarizing effects of the adjuvant alum provided a rationale for investigation of inflammasome SNPs effects on T1D risk and progression as well as efficacy of GAD-alum treatment. My thesis presents our findings concerning the role of Treg and the NALP3 inflammasome in T1D and GAD-alum treatment.

We reported increased expression of Treg-associated markers in children with recent-onset T1D as well as in children at increased risk of developing T1D. Based on results from earlier in the phase II trial of GAD-alum indicating increased Treg frequencies after antigen recall, we expanded our analysis of Treg during the 4-year follow-up of the same trial. 4 years after treatment, expansion of cells with a Treg phenotype was still evident after antigen recall, however we also reported the expansion of cells with an activated effector T cell phenotype. Furthermore we provide evidence that patients with T1D have impaired Treg-mediated suppression of Teff compared to healthy individuals, and that this impaired suppression is dependent on the Treg in the T1D population analyzed by us. We could find no evidence that GAD-alum treatment affected impaired suppression in patients with T1D. Subsequently, we monitored the frequencies of Treg, Teff, memory T cells, GAD_{65} specific T cells and GAD_{65}-induced T cell proliferation in a phase III trial of GAD-alum, and found that antigen recall induced mainly Teff and memory T cells and non-suppressive T cells throughout the trial. However, we also observed effects on subsets of Treg which have previously been reported to be capable of more potent suppressive function. We demonstrate an increase in GAD_{65} specific T cells on antigen recall after GAD-alum treatment, and provide evidence that antigen specific cells are mainly Teff and memory T cells.

Taken together, our findings do not clearly support the idea that Treg are mediators of clinical efficacy of GAD-alum. Rather it is possible that a skewing of the immune response toward a Th2-type response in contrast to the Th1-type immunity associated with T1D is involved in the mechanism of action of GAD-alum treatment, as reported by our group.

Our analyses are limited to cells obtained from peripheral blood, and might not properly represent the immunological situation in the pancreatic islets or pancreatic draining lymph nodes. While complicated, immunological analysis at these sites would be extremely valuable in elucidating the role of Treg in T1D and GAD-alum treatment. We have not yet performed suppression assays using Treg and Teff obtained from patients participating in the phase III trial to confirm our findings in Paper II in a larger population and in patients receiving 4 doses of GAD-alum, but expansion of cells and validation of a suppression assay to this end is currently ongoing.

Finally, we could find no evidence of an increased risk of developing T1D in individuals with variant alleles in 3 SNPs related to the NALP3 inflammasome, nor could we provide evidence of an effect of NALP3 inflammasome mutations on the efficacy of GAD-alum treatment, despite strong evidence that the inflammasome is required for alum adjuvanticity. In conjunction with negative results from a clinical trial of an IL-1 receptor antagonist in patients with T1D, the current data does not provide strong support for a significant role for the inflammasome and IL-1β signaling in the pathogenesis of T1D.

The phase III clinical trial of GAD-alum examined in Paper III failed to reach its primary end point, and an important question is why the clinical efficacy demonstrated in the phase II trial could not be repeated. The phase III trial was closed after 15 months, but a subgroup of Swedish patients had already completed the 30 month follow-up. Results from this subgroup indicate a clinical benefit of 2 doses of GAD-alum at 30 months after treatment. It is important to note that the phase II trial did not reach its primary endpoint at 15 months either, but clinical effects were instead demonstrated 30 months after treatment. A 4-year follow-up of the phase III trial is underway to determine whether clinical benefits can be confirmed in a larger cohort. The most pronounced positive effect on preservation of C-peptide was found in patients with shorter disease duration in the phase II trial, and thus the inclusion criteria for the phase III trial were changed to reflect this. It could be speculated that the shorter disease duration of the population could include a higher number of patients experiencing a honeymoon period, which might potentially mask early effects of GAD-alum treatment, though probably not as late into the treatment as 15 months. Furthermore, the treated groups had more patients between the age of 10 to 11 years, while the placebo group contained more patients between 16 to 20 years of age, and since the decline in C-peptide levels might be more rapid in younger patients this might obscure effects of GAD-alum treatment on C-

peptide secretion. Finally, it is currently not clear whether mass vaccinations against Influenza A H1N1 that occurred during the trial in Sweden affected the outcome of the trial.

The use of GAD-alum as an immunomodulator was based on promising results from studies in animal models where GAD treatment suppressed the progression of T1D. When trials of GAD as an immunomodulator in humans began, alum was chosen as an adjuvant in part to promote Th2 immunity thought to be protective in T1D. After mixed results of GAD-alum treatment in humans, Boettler *et al* went back to the pre-clinical setting and examined the effects of GAD-alum, in contrast to unformulated GAD used previously, in animal models. They found that while GAD-alum treatment induced a specific Th2 response in a transgenic rat model, it did not protect against diabetes [269]. This indicates that the choice of alum as an adjuvant in clinical trials might have been unwise, and that there may still be potential for use of GAD_{65} in immunomodulation in humans. It is possible that different doses, administration frequency or administration routes, or different or even no adjuvant may enhance the effect of GAD-alum treatment. It may also be more effective in a preventive setting, where a larger β-cell mass remains, and this is currently being addressed in the DiAPREV-IT prevention trial using GAD-alum. GAD-alum is also being evaluated as a combination therapy with vitamin D and ibuprofen in the DIABGAD study which is currently recruiting participants.

In conjunction with other extensive projects performed by our group, focusing on cytokine profiles and autoantibody levels after GAD-alum treatment, our data provides an important addition of knowledge to better understand the immunomodulatory effects of antigen treatment in autoimmunity.

To address the title of this thesis, it appears that the reign of the Treg is usurped in T1D, but whether this is due to incompetent rulers or rebelling subjects, and how this coup d'état is achieved, remains to be determined.

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