FUNCTIONAL GENETICS OF TYPE 1 DIABETES:
Between Genes and Disease

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1 INTRODUCTION

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an autoimmune disease in which the body's pancreatic β-cells are destroyed through a still incompletely understood immune reaction. β-Cells are highly differentiated neuroendocrine cells that are the only source of the glucose-regulating hormone insulin in the body. Autoimmune destruction of β cells leads to a deficiency of insulin and impaired glucose homeostasis, as without insulin peripheral tissues such as fat, liver and skeletal muscle cells are incapable of internalizing glucose from the bloodstream. The inability of cells to take up glucose leads to hyperglycemia, while leaving tissue cells deprived of their primary energy source. Under normal physiological conditions glucose is not present in urine, as it is completely reabsorbed in the kidneys. When levels of glucose in the blood surpass the kidney's reabsorption capacity, the excess of glucose is being excreted in the urine, causing the phenomenon from which T1D derives its name; diabetes mellitus means *honey-sweet flow* in ancient Greek. Due to the osmotic effect of glucose in the renal tubules, glycosuria causes the concomitant loss of large quantities of water and electrolytes, leading to the pathognomonic symptom of polyuria. In turn, polyuria causes dehydration, leading to the second pathognomic feature of diabetes mellitus; polydipsia, or excessive thirst. In the diabetic state glucose is no longer available as an energy source for peripheral tissues and the body reverts to alternative mechanisms of energy production, such as the oxidation of fatty acids, to maintain vital cellular processes. During the oxidation of fatty acids acidic ketone bodies are produced as a by-product. The loss of circulating volume and electrolytes that results from the glycosuria, combined with the increased production of acidic ketone bodies can lead to the potentially fatal medical emergency known as diabetic ketoacidosis. If the metabolic acidosis is not corrected swiftly using intravenous administration of fluids and exogenous insulin, the condition will lead to coma and eventually death.[1]

T1D is one of the oldest diseases known to medicine, being already described by ancient Egyptian physicians in the Eber's papyrus.[2] Despite modern medicine's familiarity with the disease, it has remained an unequivocal death sentence within months after diagnosis until 1921, when Banting and Best were the first to isolate insulin from the carcass of a dog . This breakthrough paved the way for the first actual treatment for T1D, insulin replacement therapy, converting a lethal disease into a chronic condition. Despite many advances in the treatment of T1D over the years, including the development of new insulin analogues, improvement in insulin administration and overall better healthcare, insulin replacement therapy targets the consequences, or symptoms of the disease, and not its cause. T1D patients will therefore retain a life-long dependency on exogenous insulin administration and remain continuously at risk for the acute complications of insulin replacement therapy, i.e. hypoglycemic coma and diabetic ketoacidosis, both potentially fatal conditions on their own.[3] In addition to the acute complications of their treatment, T1D patients are at risk for long-term complications such as ocular, neurological, cardiovascular and renal

complications associated with prolonged hyperglycemia, as exogenous insulin cannot match the precision of the body's own mechanisms of glucose regulation.[4-6] Further, T1D patients often experience reduced quality of life as well as a diminished life expectancy despite the best available therapies.[3] Elucidation of the exact etiology of T1D is a long sought-after goal in modern medicine as it may provide novel targets for a curative treatment or even allow prevention of disease.

Over the course of the 20th century the worldwide incidence of T1D has been on the rise and is expected to have doubled in children under the age of 5 in the time-period from 2005 to 2020.[7,8] The cause for this marked rise in incidence remains obscure, but given the short time-span in which this increase takes place genetic variability is unlikely to be accountable. Under healthy conditions the human body has an insulin production capacity in vast excess of its physiological needs and T1D only becomes clinically overt when the insulin production fails to meet the absolute minimum requirements. At time-of-diagnosis the autoimmune destruction of β-cell often has been ongoing for a long time and the majority of β-cell function is already lost. This delay poses difficulties for both research as well as potentially curative or preventative treatments as elucidating the initiating trigger for disease development long after its onset poses a major challenge. Further, β-cells do not appear to have any clinically relevant regeneration rates, reducing the chances for T1D patients to regain spontaneous insulin-independence even if the autoimmune process could be brought to a halt immediately upon diagnosis of the disease. While increases in pancreatic β-cell mass has been described in conditions of increased insulin demand, such as obesity[9] and pregnancy[10], on average these changes are limited. T1D patients often experience a temporal decrease in the need of exogenous insulin administration shortly after diagnosis, a period referred to as the honeymoon phase. While the existence of this honeymoon phase and the data obtained from obesity and pregnancy suggest that (partial) restoration of lost beta cell function is possible, there currently are no possibilities to stimulate this response and the chances for T1D patients to regain spontaneous, persistent insulin-independence are slim. Therefore, being able to predict disease occurrence by elucidating the factors that predispose to disease may facilitate both prediction and prevention of T1D.

The estimated lifetime risk for the development of T1D is estimated at 0.4%, but geographically a large variation in T1D occurrence exist. Globally, incidence ranges from less than 1/100.000 per year in China to over 50/100.000 per year in Finland.[7,11] In addition to the geographic variation T1D displays aggregation in certain families, causing an increased risk of T1D development in individuals with affected parents, siblings or offspring.[12-14] Children with an affected first-degree family member have a lifetime risk of 3-8% to develop T1D, but also parents have an increased risk for developing the disease themselves as soon a one of their children becomes affected. The actual incidence in individuals with an affected first-degree family members depends on many factors, among which, but not limited to, ethnicity, family member affected, the age of diagnosis for the affected family member, birth order and maternal age of delivery, the HLA-type of

the affected family member and the degree of genetic similarity with the affected family member.[15-18] In general, children with a diabetic mother have an estimated risk of 1-4% for the development of T1D, while those with an affected father have a lifetime risk of 3-8%. This inherited parental risk is multiplicative, rather than additive, and when both parents are affected the lifetime risk for their children to develop T1D can be as high as 30%. In the case of affected siblings, the a-priori risk for disease development ranges from 3-6% in case of non-twin siblings, to 8% in dizygotic twins and 30-65% in the case of monozygotic twins.[19] The proband-wise concordance rate of monozygotic twins is high, but no complete, despite their identical genetic makeup. Although the concordance rates increase with the number of genetic risk factors present, it does not become complete, suggesting that factors other than genetic variation play a role in T1D development.[20-22] This is indicated as well by the effects of birth order and maternal age at birth, which are difficult to explain from a genetic viewpoint, and the fact that the vast majority (>90%) of new T1D cases occur in individuals without a family history of the disease. These, in addition to genetic susceptibility other factors must be involved in the onset of β cell autoimmunity. Environmental factors such as dietary intake of vitamin D, cow's milk and gluten as well as increased hygienic conditions and viral outbreaks have all been implicated as risk modulators, classifying T1D as a complex disease with both genetic and environmental factors interacting in its pathogenesis.[23-27]

Immunopathology of type 1 diabetes

During evolution, the immune system has adapted to protect the body against a plethora of possible pathogens such as viruses and bacteria while leaving one's 'self'' unharmed. The adaptive immune system, composed of B and T lymphocytes, is capable of mounting highly effective, antigen specific immune responses against a broad range of different antigens. This broad reactive capacity results from rearrangement of genetically encoded receptor subunits during T and B cell development, which are linked together using a random sequence of nucleotides.[28] The addition of random nucleotide insertions creates a highly diverse pool of T and B cell receptors, required to combat the many pathogens one might encounter. Yet, it also allows for the generation of immune cells with the propensity for autoreactivity. The removal of these potential deleterious immune responses from the pool of immune cells during T cell development is enforced in a process referred to as induction of central tolerance.[29]

T cells undergo an essential part of their development in the thymus, where specialized medullary thymic epithelial cells (mTECs) express and present self-antigens under the governance of the autoimmune regulator gene *AIRE*.[30] T cells recognizing self-derived epitopes on the surface of mTECs commit to apoptosis or become anergic, rendering them inert, hereby theoretically clearing the T cell pool of all autoreactive cells. Yet, as demonstrated by the existence of various autoimmune diseases, this process of negative selection is not flawless. The mechanisms by which potentially autoreactive T cells escape thymic education are not clear, but suboptimal antigen presentation due to low binding-

affinity of various self-peptides to human leukocyte antigen (HLA) is implicated. [31,32] Alternatively, potential autoreactive T cells can express low avidity T cell receptors, causing them to survive negative selection despite interacting with self-peptide:HLA complexes on the surface of mTECs.[33] A third possibility entails the absence of the cognate autoantigen from the thymus, either due to differential expression between thymus and peripheral tissue or as a result of tissue-specific posttranslational modifications, causing a discrepancy between periphery and thymus.[34,35] It is conceivable that different mechanisms are involved for different autoantigens. Determining the method of tolerance evasion for each islet-autoantigen in T1D will help focus therapeutic interventions as each method of tolerance evasion will require its own, tailored treatment approach. Currently, the escape of autoreactive T cells from the thymus is viewed as deleterious, yet islet-reactive T cells are not exclusive to T1D patients but can be readily detected in the peripheral blood of in healthy individuals as well. Therefore, determining the actual consequences of thymic escape by analysing these cells in terms of phenotype and function, may help distinguish autoreactive T cells involved in disease propagation from "bystander" autoreactive T cells, that pose a distraction in the elucidation of disease etiology .

To restrain the autoreactive cells that have eluded thymic selection, a second mechanism of tolerance exists, known as peripheral tolerance. Regulatory T cells (Tregs), which repress rather than promote immune activation upon activation, are pivotal in this process of peripheral tolerance. Tregs can be either naturally occurring Tregs, (nTreg), which suppress immune responses in a non-antigen-specific manner, or induced suppressor cells (iTregs) which act in an antigen-specific manner. In T1D a diminished functionality of Tregs as well as increased resistance of autoreactive effector cells to Treg suppression have been reported, contributing to the immune dysregulation required for autoimmune diseases to occur [36,37]. The actual mechanism by which autoreactive T cells in T1D increase their resistance to regulation by Tregs remains unknown thus far and its elucidation may offer opportunities to correct this imbalance and revert aberrant immune responses, or even prevent them from ever happening at all.

Several events have been proposed to precipitate autoimmunity in T1D by creating immunological "danger signals" that attract autoreactive cells to the site of the pancreas, including viral infections, environmental conditions leading to endoplasmic reticulum stress or bacterial transposition from the small intestine. [38-41] Yet, at present the trigger for the initiation of β-cell destruction remains unknown. After the initial autoantigen-presentation to the immune system and subsequent immune activation that lead to β cell destruction, islet-reactive T cells with different specificities that the original culprit can be activated in a process called epitope spreading.[42] In addition, unrelated T cells can get activated in a non-antigen specific manner via the process of bystander activation.[43,44] Under normal circumstances both these mechanisms are beneficial as they add to improved clearance of pathogens, however they pose a cardinal problem in the elucidation of T1D pathogenesis as T1D is often diagnosed long after the initial onset of autoimmunity. The search for the causative agent in T1D development is further complicated by the great

degree of heterogeneity of disease progression found among T1D patients, which suggests that perhaps there is no "single" disease mechanism but rather a multitude of disease processes with a common final pathway.[45] Theoretically, any abrogation of immune regulation, such as impaired expression of immune-regulatory molecules or inadequate maintenance of autoreactive immune cells, may eventually lead to loss of tolerance and consequently start the cascade of autoimmunity that leads to T1D.

Current evidence suggests that both $CD4^+$ and $CD8^+$ T cells play a pivotal role in the process of β-cell destruction.[46] CD4+ T cells recognize extracellular antigens and promote local inflammation by releasing cytokines, whereas CD8+ T cells recognize peptides of intracellularly synthesized antigens on the surface of their target cells, which they subsequently lyse upon engagement. The specific destruction of insulin producing β-cells in T1D, sparing adjacent neuroendocrine islet cells such as glucagon-producing α-cells and somatostatin-producing δ- cells, implies that β-cell-specific antigens are targeted during the autoimmune response of T1D. Reactivity against various islet antigens has been described for T1D, yet no single islet autoantigen has unequivocally been identified as driving the autoimmune process in type 1 diabetes.[45,47] In addition to T cells, members of the innate immune system have been implicated in T1D development.[48] Dendritic cells are crucial in the initial antigen -presentation to autoreactive T cells[49], whereas macrophages and NK cells act as sources of pro-inflammatory cytokines and chemokines. In addition, NK cells can directly interact with β-cells leading to their demise.[50-52] Several pro-inflammatory cytokines produced by the innate immune system have been implicated directly in β-cell death, among which most notably interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α). β-cells appear more sensitive to the apoptotic stimulus provided by TNF-α and IL-1B than α- and δ-cells, possibly contributing to the selective loss of insulin producing cells.

The first steps towards the discovery of antigenic targets in T1D were made in 1974 with the indirect immunostaining of pancreatic tissue with islet cell antibodies from the serum of polyendocrine-disease patients.[53] To date, multiple β-cell antigen have been found to be autoantibody targets among which (prepro-)insulin, the 65 kDa isoform of glutamic acid decarboxylase (GAD65), insulin autoantigen 2 (IA2), carboxypeptidase H, zinc transporter 8 (ZnT8), imogen-38 and phogrin.[54-60] In addition, several self-antigens have been found to be targeted exclusively by T lymphocytes and not antibodies, including islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), islet amyloid polypeptide ppIAPP and heat shock protein 60 (hsp60).[61-63] While pivotal in the initial discovery of several autoantigens in T1D, the role of autoantigens in disease propagation of T1D remains controversial and presently autoantibodies in T1D are viewed as markers of immune activation and disease progression rather than causal agents in destruction of β-cells. This view is strengthened by reports of T1D development of in the setting of B-cell deficiency.[64] Notwithstanding, anti B-cell therapies have been shown to temporarily delay the loss of insulin production in T1D in a small group of patients, indicating that the precise role of autoantibodies in the disease process of T1D remains to be determined.[65,66] Despite

the fact that recent years have provided renewed understanding of key immunological players involved in β-cell destruction (Figure 1), the precise reason why tolerance is lost against β-cell antigens in type 1 diabetes remains obscure to date.

Genetic contribution to T1D susceptibility

As described previously, the familial clustering of T1D has long suggests involvement of genetic factors in T1D susceptibility. Proof hereof came with the association of the human leukocyte antigen (HLA) locus and T1D development.[67-69] The HLA complex, or major histocompatibility complex (MHC), is a highly polymorphic gene locus containing more than 120 genes, over 40% of which are believed to be involved in immune processes.[70,71]

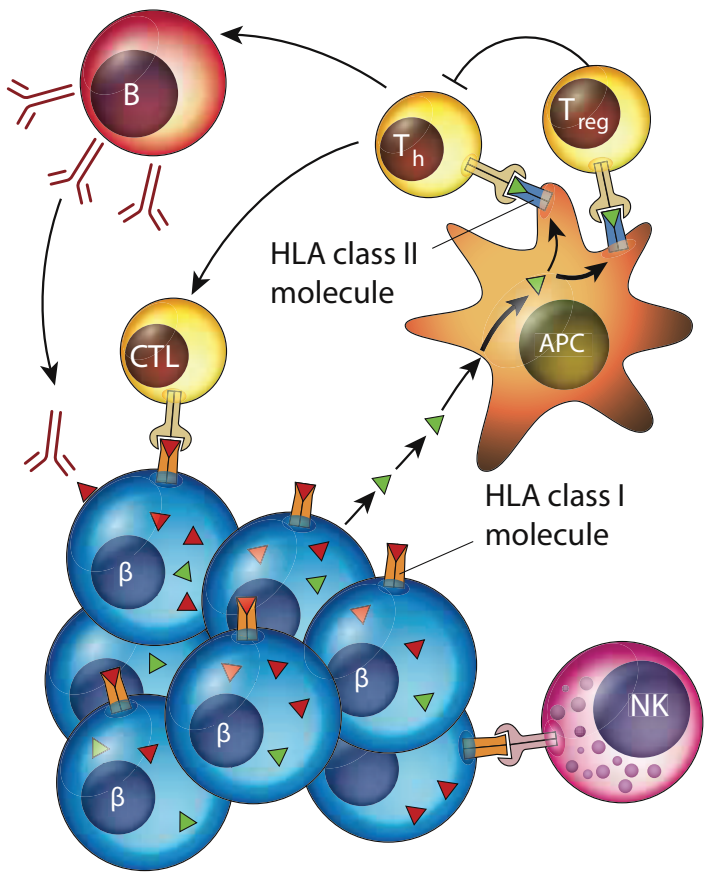

Figure 1. The current view on islet autoimmunity in Type 1 Diabetes. The pathogenesis of type 1 diabetes revolves around the presentation of islet antigens to $CD4^+$ T cells by professional antigens presenting cells. $CD4^+$ T cells propagate the immune response by activating $CD8^+$ and B cells. Once primed, $CD8^+$ T cells become licenced to kill β-cells. NK cells can directly interact with β-cells and together with other constituents of the innate immune system act as source of (potentially β cell toxic) cytokines and chemokines. (B.O. Roep, Nature, 2007 [103])

The most important regions of the MHC for T1D are the HLA class I and II genes that encode surface molecules involved in presenting intracellular peptide fragments to T lymphocytes, with the greatest risk modifier for T1D development mapping to the HLA class II locus.[72] CD8+ cytotoxic T cells recognize their cognate antigen in the context of MHC class I, whereas CD4+ T cells recognize antigen in the context of class II molecules. Genetic fine mapping has revealed that polymorphic DR and DQ α-β heterodimers, encoded by the *HLA-DQB1* and *HLA-DRB1* genes, are central to disease susceptibility.[73] Due to the high degree of linkage disequilibrium within the HLA locus, certain alleles often occur together and are referred to as haplotypes. Two specific haplotypes carry the greatest risk for T1D, i.e. DR3 (DRB1*03:01-DQA1*05:01-DQB1*02:01) and DR4 (DRB1*04:01-DQA1*03:01-DQB1*03:02).[74] Over 90% of all T1D patients express either DR3 or DR4, while only 40% of non-affected carry either haplotype.[75,76] Separately, DR3 and DR4 increase susceptibility for T1D (odds ratios of 3.6 and 11.4, respectively), yet their risks act synergistically and individuals heterozygous for HLA-DR3/DR4 have up to a 47-fold increased risk compared to the general population.[72] This extreme risk haplotype is present in 2.3% of United States-born Caucasian children, while 39% of patients who develop T1D before the age of 20 have this specific genotype.[77] Other HLA haplotypes increase the risk of type 1 diabetes to a lesser extent, while some haplotypes even seem to protect against developing this condition. The DR15-DQ6 (DRB1*15:01- DQA1*01:02-DQB1*06:02) haplotype is present in ~20% of the general population but only in 1% of T1D patients and has a dominant protective association with T1D with and odds ratio of 0.03.[72,78] Due to a high degree of linkage disequilibrium it has been difficult to determine other, weaker, genetic risk factors within the HLA locus. Still, the independent effect of several HLA class I genes on T1D susceptibility, most notably HLA-B*57:01 and HLA-B-39:06, was discovered recently, [79,80]. The mechanism by which HLA alleles influence T1D risk is not completely clear, but it is presumed that the risk conferred by these alleles and haplotypes relates to specificity of self-peptide binding and presentation.[47,81]

In addition to the HLA locus 58 non-HLA regions have been found to associate with T1D development.[82-85] In contrast to the HLA region, which is thought to account for ~50% of all genetic risk in T1D, the majority of these T1D risk loci only carry a moderate risk for disease development (OR 1.1~1.5).[83,84,86] Among the first non-HLA regions discovered to associate with T1D are the *INS* locus, harboring the gene coding for insulin, and the *CTLA4* locus which codes for the immune-regulatory surface receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[87,88] Compared to other known non-HLA risk regions, these regions carry a relatively large genetic risk which might explain why their associated genetic risk for T1D susceptibility was be detected in relatively small patient studies prior to the large cohort genome-wide associated studies (GWAS). The *INS* locus became a premier candidate for genetic association with T1D due to insulin's central role in glucose homeostasis and its unique distinction as the only known β cell-specific antigen. Evidence for genetic linkage of the insulin gene with T1D came when variation of a variable number of tandem repeats (VNTR) upstream of the *INS* gene was shown to associate with risk

for T1D development.[89-91] Alleles with short (class I) VNTR elements predispose to T1D, while longer (class III) VNTR alleles are dominantly protective. The length of the VNTR influences expression of the INS gene in *cis*, with class III alleles causing a 20% reduction of insulin expression in the pancreas, but up to threefold higher expression in the thymus compared to class I alleles.[3,92] It is hypothesized that the increase in thymic insulin expression facilitates negative selection of autoreactive cells, thereby diminishing the risk for T1D development.[92,93] Overall, the role of the *INS* locus can be viewed as prototypical for the role of the thymus in the development of autoimmune diseases, where proper education of T-cell is critical in regulating autoimmunity. The discovery that the genetic risk of the *INS* region was linked to thymic expression of the autoantigen prompted further investigation and a role for differential thymic expression in the occurrence of autoreactivity was postulated for additional islet-autoantigens in T1D, i.e. islet antigen 2 (IA-2) and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP). The latter will be discussed in chapter 2 of this thesis.

The *CTLA4* region was the third genetic locus to be associated with T1D predisposition. The *CTLA4* gene encodes a receptor on the cell surface of T lymphocytes that negatively regulates co-stimulation of T cells, by binding the same B7 ligand as the activating T cell receptor CD28.[94] In contrast to CD28, the intracellular part of CTLA-4 has phosphatase activity that downregulates the intracellular signals that take place after the T-cell receptor engages with its cognate HLA:peptide complex and thereby inhibits immune activation. The *CTLA4* gene was first shown to be associated with the risk of T1D in several candidate gene study and more recently GWAS have confirmed the association of the *CTLA4* locus with not only T1D, but also rheumatoid arthritis, celiac disease and autoimmune thyroid disease.[95-97] The association of *CTLA4* polymorphisms with multiple autoimmune diseases underlines the importance of its immune regulatory capacities. It is conceivable that polymorphisms within the *CTLA4* region lead to altered expression or functionality of CTLA-4 and thus aberrant immune regulation. Yet, despite fine-mapping of the genetic risk to a single nucleotide polymorphism (SNP) located near the 3' end of the *CTLA4* gene, to date no actual functional implications have been attributed to this polymorphism. The potential of CTLA-4 modulating agents in treatment of autoimmune disease has recently been indicated by a clinical trial with abatacept, a CTLA-4 fusion immunoglobulin, that showed a temporary delay of β-cell loss in treated recent onset T1D patients. Thus, elucidating the mechanism behind the genetic association of the *CTLA4* region with autoimmune diseases may provide more novel therapeutics and tailored treatment strategies .

Many of the T1D-associated non-HLA loci have only recently been discovered through comprehensive GWA studies in which large cohorts of T1D patients and matched controls are analyzed for over 500,000 different SNPs, covering the whole human genome.[83] The vast majority of the newly identified risk regions has at most a modest impact on disease susceptibility, reflected by low odds ratio (varying from ~1.1 to ~2.0), and many of the associated risk variants are common in the generic population. While many regions

harbor genes with immunological functions and show similarity to the *CTLA4* locus as they associate with susceptibility for multiple autoimmune diseases, for the majority of associated regions no validated causal gene has been identified. Further, for those loci with validated causal genes the mechanisms through which these genetic variations influence disease remains unclear. It is estimated that taking all currently known disease-associated genetic variation into account approximately 40-88% of the observed familial clustering, or heritability, of T1D can be explained.[73,98-100] The remaining 'missing heritability' is thought to be caused by genetic variations that are not investigated in GWA studies, such as deletions, insertions and copy-number variants in addition to rare genetic variants with a frequency too low to properly detect in GWA studies. Additionally gene-gene and gene-environment interactions may modulate the risk of genetic variants, and these effects cannot be directly investigated with genomic screening.[101] Environmental triggers may alter gene transcription through epigenetic modifications such as DNA-methylation or alterations of the histone code[102]. Alternatively, environmental changes can influence the expression small non-coding RNAs, e.g. microRNAs, which regulate gene expression at a post-transcriptional level. Identification of the 'missing genetic risk' and elucidation of the pathways by which disease associated genes contribute to disease development is crucial for the clarification of T1D pathogenesis. Genetic predisposition undeniably contributes to disease occurrence, yet by itself it is not sufficient for disease development as demonstrated by the incomplete concordance of affected twin pairs and the discrepancy between occurrence of very-high risk genotypes versus disease prevalence. Additional immune dysregulation appears to be required to 'hot-start' the autoimmune destruction of β-cells. Identifying factors that modulate genetic risk through gene-environment interactions might reveal potential triggers preceding disease development and may lead to new therapeutic modalities and strategies of disease prevention.

AIMS OF THIS THESIS

Identification of the cellular mechanisms involved in the occurrence and persistence of autoreactive lymphocytes is key for understanding T1D etiology. Comparing autoreactive T lymphocytes from healthy individuals and T1D patients can provide clues as to what the driving force is for the destruction of β-cells and might designate potential targets for (immune) intervention. Elucidating in what way T1D associated gene variants actually contribute to disease development, i.e. understanding the functional aspects of genetic risk, and how genetic control of autoantigens influences autoimmunity may provide crucial clues to the clarification of the enigma of T1D. This thesis aims to answer several of these issues by investigating the role of transcriptional and post-transcriptional gene control in T1D.

In **chapter 2** the immunological implications of differential expression and splicing of *G6PC2*, the gene coding for the islet-autoantigen IGRP, and the role of thymic education on the occurrence of IGRP reactive lymphocytes is discussed. In **chapter 3** we compare

IGRP-reactive T lymphocytes between health and disease and identify a major difference in proliferative capacity that associates with differential microRNA-mediated regulation of the pro-apoptotic FAS and TRAIL pathways in diabetogenic T lymphocytes. **Chapter 4** of this thesis discusses the phenotypic effects that rare genetic variants located within known T1D risk genes can exert by affecting the post-transcriptional control of microRNAs, while **chapter 5** provides evidence that the $(AT)_n$ microsatellite in the *CTLA4* 3'UTR is causal for the association of the *CTLA4* with T1D susceptibility via reduced CTLA-4 expression in lymphocytes of individuals carrying susceptibility alleles.

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2

ALTERNATIVE SPLICING AND DIFFERENTIAL EXPRESSION OF THE ISLET-AUTOANTIGEN IGRP BETWEEN PANCREAS AND THYMUS CONTRIBUTES TO IMMUNOGENICITY OF PANCREATIC ISLETS BUT NOT DIABETOGENICITY IN HUMANS

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SUMMARY

Thymic expression of self-antigens during T-lymphocyte development is believed to be crucial for preventing autoimmunity. It has been suggested that *G6PC2*, the gene encoding islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), is differentially spliced between pancreatic beta cells and the thymus. This may contribute to incomplete elimination of IGRP-specific T lymphocytes in the thymus, predisposing individuals to type 1 diabetes. We tested whether specific splice variation in islets vs thymus correlates with loss of tolerance to IGRP in type 1 diabetes. Expression of *G6PC2* splice variants was compared among thymus, purified medullary thymic epithelial cells and pancreatic islets by RT-PCR. Differential immunogenicity of IGRP splice variants was tested in patients and healthy individuals for autoantibodies and specific cytotoxic T lymphocytes using radiobinding assays and HLA class I multimers, respectively. Previously reported *G6PC2* splice variants, including full-length *G6PC2*, were confirmed, albeit that they occurred in both pancreas and thymus, rather than islets alone. Yet, their expression levels were profoundly greater in islets than in thymus. Moreover, three novel *G6PC2* variants were discovered that occur in islets only, leading to protein truncations, frame shifts and neo-sequences prone to immunogenicity. However, autoantibodies to novel or known IGRP splice variants did not differ between patients and healthy individuals, and similar frequencies of IGRP-specific cytotoxic T lymphocytes could be detected in both patients with type 1 diabetes and healthy individuals. We propose that post-transcriptional variation of tissue-specific self-proteins may affect negative thymic selection, although this need not necessarily lead to disease.

INTRODUCTION

Type 1 diabetes is characterised by the autoimmune destruction of insulin-producing beta cells in the pancreas. Autoreactive cytotoxic T lymphocytes (CTLs) are pivotal in the actual beta cell destruction and type 1 diabetes development. While the majority of autoreactive T cells are thought to be deleted during their development in the thymus[1], the process of thymic education appears inherently incomplete, as islet-reactive T cells can be readily detected in peripheral blood of both patients with type 1 diabetes and healthy individuals.[2-4] Proper negative selection of autoreactive T cells is dependent on the presentation of peripheral tissue antigens by medullary thymic epithelial cells (mTECs) and thymic dendritic cells under the control of the transcription factor autoimmune regulator (AIRE).[5,6] T cells recognising self-peptide presented by thymic dendritic cells or mTECs with high avidity are removed from the T cell repertoire through apoptosis. However, T cells recognising autoantigens with low avidity[7,8], or T cells reactive against self-epitopes that are not expressed in the thymus, may evade negative selection and escape into the periphery.[9]

Several islet antigens have been proposed as antigenic targets for autoreactive CTLs in type 1 diabetes development, among which is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP).[10-12] IGRP is a 355-amino acid endoplasmic reticulum-embedded phosphatase that plays a role in glucose homeostasis.[13,14] Increased reactivity against IGRP has been observed in patients with recent-onset type 1 diabetes, implying involvement of IGRP-reactive T cells in the development of human type 1 diabetes.[3,15] The gene coding for IGRP, *G6PC2*, can give rise to multiple splice variants, and it has been hypothesised that the incomplete tolerance to IGRP is caused by differential expression of splice variants between the thymus and pancreatic islets.[16] Notably, *G6PC2* transcripts containing exons 3 and 4, including full-length *G6PC2*, have been reported to be absent from the thymus and suggested to be involved in the incomplete tolerance towards IGRP in type 1 diabetes.

Here, we investigated whether differential expression of *G6PC2* splice variants between the thymus and pancreas indeed contributes to incomplete negative selection and increases the risk of disease development. For this, expression of *G6PC2* in purified mTECs, whole fetal thymus and pancreatic islet cells was analyzed. Next, we tested for humoral immunogenicity of IGRP isoforms in 60 type 1 diabetes patients and 60 matched healthy individuals. Finally cellular immunogenicity of G6PC2 splice variants was tested in peripheral blood of recent onset type 1 diabetes patients and healthy HLA-matched subjects using quatum-dot (Qdot) labeled peptide-MHC (pMHC) multimers.

METHODS

Tissue samples

Purified mTECs were obtained by 24 h incubation of neonatal tissue with dexamethasone. Snap-frozen neonatal thymic tissue was obtained from neonates requiring cardiac surgery,

as described.[17] Human islets were obtained from the islet isolation core at the Leiden University Medical Centre and stored in Trizol upon isolation. After informed consent had been given, heparinised blood samples were obtained from type 1 diabetic patients in the first year after diagnosis, and all samples were treated identically according to standard procedures. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Isopaque density gradient centrifugation; they were then frozen and kept in liquid nitrogen until use. HLA-A2 (HLA-A*0201) typing was confirmed by flow cytometry using FITC-conjugated HLA-A2 antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The study was approved by the ethics committees of all centres involved.

Detection of splice variant

Cells and tissue samples were lysed using 1 ml Trizol, and total RNA was obtained according to the manufacturer's protocol. Total RNA was treated with DNAse (Qiagen, Venlo, the Netherlands) for 45 min at room temperature to remove potential genomic DNA carry-over. RNA was subsequently cleaned using RNAeasy columns (Qiagen). First-strand cDNA synthesis was performed using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) in combination with oligo-dT primers.

RT-PCR amplification was performed using 0.4 µmol/l sense and anti-sense primer, 0.2 mmol/l dNTPs (Promega, Madison, WI, USA), 1.5 mmol/l $MgCl_2$ (Promega) and 1 U GoTaq Flexi DNA polymerase (Promega) in 25 µl reaction volume. Initial denaturation was carried out at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. Unless stated otherwise, 10 ng cDNA template was used per reaction.

Sequencing templates were generated using 1 U Pfx50 DNA Polymerase (Invitrogen, Breda, the Netherlands) with an elongation temperature of 68°C. Other conditions were identical. The nucleotide sequence of all splice variants found was confirmed by polyacrylamide gel sequencing using the Thermo Sequenase Primer Cycle Sequencing kit (GE Healthcare, Zeist, the Netherlands) according to the manufacturer's instructions.

Radiobinding assay

Sera from 60 recent-onset islet antibody-positive type 1 diabetic patients and 60 age- and sex-matched controls (mean age 12 years, range 0–34 years; male/female ratio 1.7) were obtained. *G6PC2* transcripts were subcloned into the pTNT (Promega, Leiden, the Netherlands) backbone using XhoI and NotI restriction sites using T4 DNA ligase (Promega) overnight at 16°C. Transcripts were translated in the presence of [^{35}S]methionine using the TNT in vitro translation system (Promega). Goat anti-human IGRP antibody G-16 (sc-33472; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:50 dilution as a positive control. Tracer purification and radiobinding assays were performed as previously described.[18] Results were expressed as % tracer binding. Reference serum from a healthy non-diabetic individual was included in each radiobinding assay. Tracer binding

was calculated as $(cpm_{sample} - cpm_{reference})/cpm_{total} \times$ dilution factor for each sample. Cold target inhibition with unlabelled in vitro translation products leading to loss of signal was performed for each target, confirming assay specificity.

Peptides

Peptides were synthesised using solid-phase Fmoc chemistry. All peptides were analysed by reverse phase HPLC (purity >85%) and matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) MS to confirm the expected mass. Peptide binding to HLA-A2 was tested as described.[19] IC_{50} values were calculated using nonlinear regression analysis.

Generation of pMHC monomers and Qdot labelling

Generation of pMHC monomers was performed as described previously.[20] Briefly, exchange reactions were performed by exposing UV-sensitive pMHC monomers (2 µmol/l in PBS) to UV light (366 nm UV lamp; Camag, Berlin, Germany) in the presence or absence (negative control) of exchange peptide (200 µmol/l) for 60 min. Multimeric pMHC complexes were produced by addition of streptavidin-conjugated Qdot-585, -605, -655, -705 and -800 (Invitrogen) to achieve a 1:20 streptavidin–Qdot/biotinylated-pMHC ratio.

PBMC staining with Qdot-labelled pMHC multimers

PBMC staining was performed as previously described.[7] Briefly, PBMCs (2×10^6) were stained with 0.1 µg of each specific IGRP multimer in PBS/0.5% BSA for 15 min at 37°C. Subsequently, allophycocyanin (APC)-labelled anti-CD8 and FITC-labelled anti-CD14, -CD20, -CD4, -CD40 and -CD16 (all from BD Biosciences, Breda, the Netherlands) were added for 30 min at 4°C. After a wash, cells were resuspended in PBS/0.5% BSA containing 7-aminoactinomycin D (7-AAD) (eBioscience, San Diego, CA, USA) to exclude dead cells and analysed using an LSRII flow cytometer (BD Biosciences).

RESULTS

Detection of novel G6PC2 splice variants

To test the extent of differential splicing between thymus and pancreas, the expression of *G6PC2* was assessed in purified mTECs, whole thymic tissue and purified pancreatic islet cells by RT-PCR. Primers were designed annealing to exon 1 and exon 5 of *G6PC2* such that all known splice variants could be amplified in a single reaction. In addition, we designed a panel of primers annealing to specific exon–exon boundaries to selectively amplify specific splice variants, thereby enabling us to distinguish splice variants with similar transcript lengths (Supplementary material Table 1).

Using equal amounts of input cDNA, we observed a distinct and consistent splicing pattern of *G6PC2* in islets, but not in purified mTECs or whole thymus material (Figure 1a). Weak expression of *G6PC2* was detectable in purified mTECs, suggesting that thymic expression was close to the detection limit of our assay. Indeed, when we

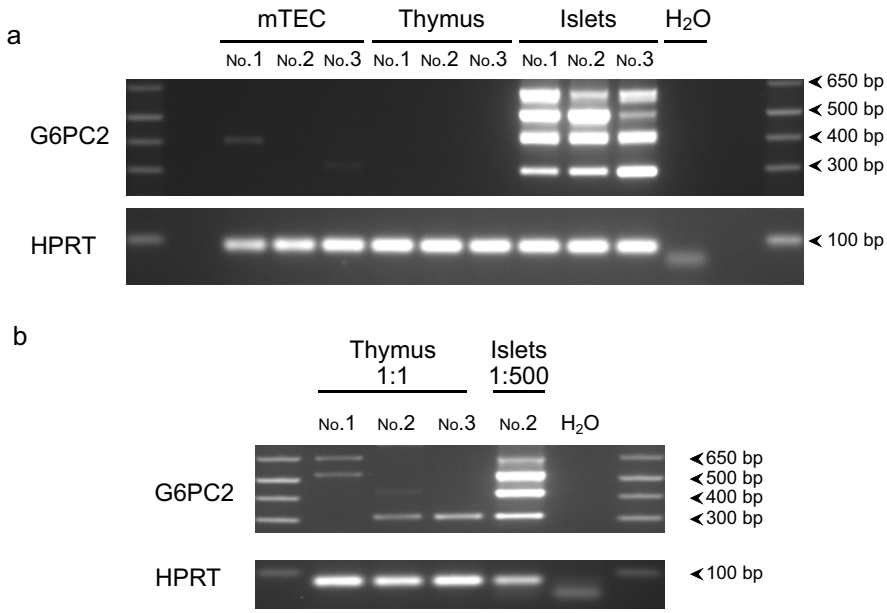

Figure 1. Expression of *G6PC2* in thymus and pancreas. (a) *G6PC2* expression was analysed by RT-PCR in separate purified preparations of mTECs and whole thymus obtained after cardiac surgery and pancreatic islets of non-diabetic organ donors. (b) RT-PCR analysis of *G6PC2* in thymus using 250 ng cDNA template. A 0.5 ng cDNA template of islet preparation No. 2 was analysed in parallel for semiquantitative comparison. The housekeeping gene hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) was used as the reference gene

increased the amount of thymic RNA per RT-PCR, thymic expression of full-length *G6PC2* and all splice variants became detectable (Figure 1b). Yet, in contrast with pancreatic islets, *G6PC2* levels in thymus were low and displayed inconsistent patterns between thymic samples from different donors. Parallel analysis of a single thymic sample yielded a stochastic pattern, with different isoforms detectable in separate RT-PCRs, confirming that thymic expression of *G6PC2* remained near the detection limit of our assay despite optimisation of input material. Nevertheless, in every thymic sample, all isoforms could be detected, albeit after specific targeting by selective PCR primers in separate analyses (data not shown).

In addition to previously reported 'conventional' splice variants that use conserved exon–exon boundaries, three novel *G6PC2* isoforms (variant A, B and C) were identified exclusively in islets. Sequencing of these isoforms showed that these variants resulted from non-conventional splicing of exon 1 (partial) and exon 5 (partial) (Fig. 2). Expression of these three non-conventional isoforms was confirmed using exon-junction-specific primers in three separate islet preparations. Furthermore, we confirmed all previously reported conventional *G6PC2* isoforms and identified two conventional isoforms not previously described lacking exon 2 or exon 3 only (Δ2 and Δ3; Supplementary Figure 1).

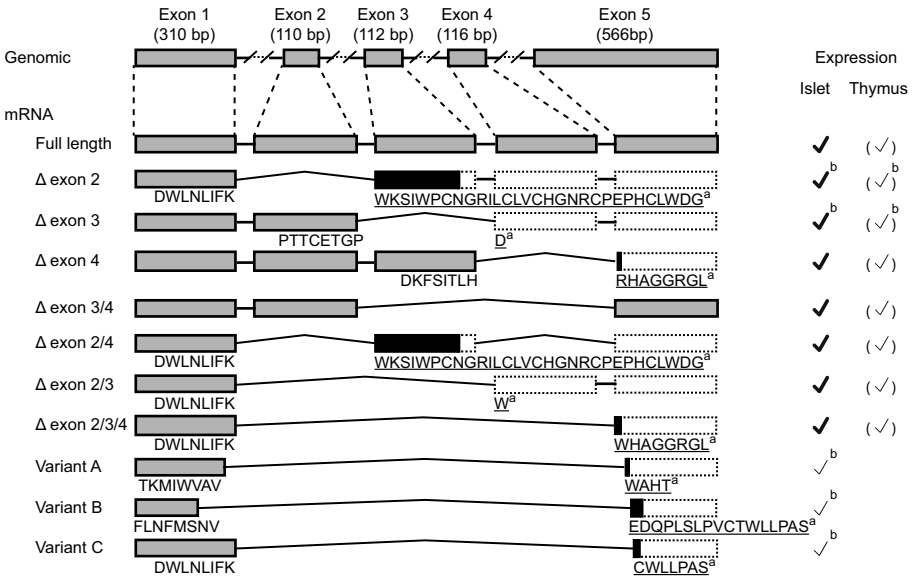

Figure 2. Overview of *G6PC2* transcripts and putative protein sequences. Exonic sequences of all *G6PC2* splice isoforms in-frame (grey), in alternative reading frame (black) and truncated (dotted). Putative neo-sequences are underlined, and in-frame protein sequences are displayed in regular font. Relative expression levels of transcripts indicated on the right: abundant (bold), low (regular) and stochastic (in parentheses). [a]Stop codon; [b]novel splice variant

Next, quantitative PCR analysis was performed to assess the relative expression of each isoform in the same pancreatic islets analysed by RT-PCR (primer sequences are specified in Supplementary Table 2). Expression of splice variants differed slightly between islet preparations, yet all isoforms could be readily detected (Supplementary Figure 2). However, as previously observed, expression of the novel splice variants, A, B and C, was low compared with other isoforms. Given that expression in thymus was detectable only around the detection limit, we decided against quantitative PCR approaches for thymic expression of *G6PC2*, as these would not add to the data already obtained. To confirm the specificity of our assay for the islet-restricted *G6PC2*, we analysed total RNA of kidney and liver, both known to express high levels of the homologous glucose 6-phosphatase (*G6PC*) in parallel. The specificity of the assay was confirmed by the absence of all isoforms in both kidney and liver (data not shown).

Detection of splice variant specific IGRP antibodies

To assess humoral immunogenicity of IGRP, the presence of autoantibodies directed against full-length IGRP as well as specific splice isoforms was tested in 60 recent-onset type 1 diabetes patients and 60 sex- and age-matched healthy individuals using a radiobinding assay. ^{35}S-labelled forms of full-length IGRP, the splice variant lacking exons 3 and 4 (Δ3/4, reported to be the most abundant isoform in thymus[16]) and the islet-specific isoforms A,

B and C were created through cell-free in vitro translation. In addition to these naturally occurring isoforms, an artificial construct coding for *G6PC2* exons 3 and 4 was created to directly assess the immunogenicity of these exons. In vitro translation of all tested isoforms into protein was confirmed using gel electrophoresis (Supplementary Figure 3).

Antibody reactivity was observed for all tested splice variants, but binding of IGRP autoantibodies was comparable between type 1 diabetes patients and healthy individuals (Figure 3). Mean antibody titres did not differ among the naturally occurring isoforms or between natural isoforms and the artificial exon 3–4 construct. Autoantibody reactivity against the islet-specific isoforms A, B and C was not significantly increased compared with isoforms expressed in thymus, nor did reactivity against these variants discriminate type 1 diabetes patients from healthy participants.

Detection of IGRP specific CD8$^+$ T-cells

G6PC2 can give rise to at least 10 splice isoforms in addition to full-length IGRP (Figure 2). All these isoforms may theoretically act as neo-antigens not present in full-length IGRP, as they emerge either through novel exon–exon junctions or shifted reading frames as a result of alternative splicing. Expression of *G6PC2* splice variants encoding unique

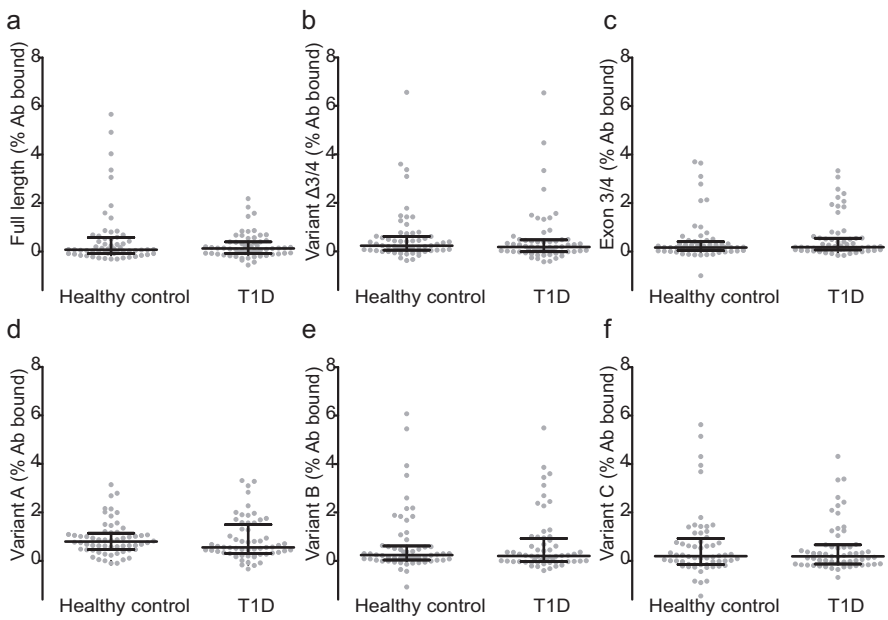

Figure 3. Antibody reactivity against IGRP isoforms in type 1 diabetic patients and healthy individuals. Radiobinding assays were performed for (**a**) full-length IGRP, (**b**) variant Δ3/4, (**c**) exon 3/4, (**d**) variant A, (**e**) variant B and (**f**) variant C. Antibody (Ab) binding was determined as the percentage of radioactive tracer that immuno-complexed with human serum (see Methods). T1D, type 1 diabetes. Statistical analysis was performed using the Mann–Whitney *U* test. Median values plus interquartile range (IQR) are displayed

amino acid sequences in pancreas, but not thymus, may lead to autoimmune targeting of beta cells. To test whether IGRP peptides contribute to cellular autoimmune responses in type 1 diabetes, all putative amino acid sequences of IGRP variants were tested in silico for high-affinity binding to HLA-A2*0201. We particularly focused on peptides derived from exons 3 and 4, those generated by novel exon–exon boundaries, and peptides from the islet-specific isoforms variants A, B and C. Peptides derived from exon 1, present in all thymic splice isoforms, were included in the search to compare relative immunogenicity of splice isoforms present in thymus with those with a presence limited to pancreatic islets (Table 1). All peptides with predicted HLA-A2 binding were synthesised, tested for actual in vitro binding, and used to generate HLA multimers as previously described.[20]

PBMCs of HLA-A2-positive type 1 diabetes patients and healthy controls were stained with IGRP-specific pMHC multimers in a combinatorial fashion to detect $CD8^+$ T cells against multiple epitopes simultaneously in a single sample, increase staining sensitivity, and reduce background signal. As a negative control, pHLA multimers were used that were UV-exchanged in the absence of rescuing IGRP peptide and Qdot-labelled in the same manner as IGRP pHLA multimers. CTLs against a broad range of IGRP peptides were observed that were comparable between recent-onset type 1 diabetes patients and healthy individuals (Figure 4). Frequencies of CTLs recognising peptides from the proposed high-immunogenic exons 3 and 4 (proposed to be lacking in the thymus) were as common as those recognising the supposedly less immunogenic exon 1. T cells recognising IGRP isoforms exclusive to pancreatic islets (variants B and C) could be detected at the highest

Table 1. Predicted HLA-A2*0201 binding peptides derived from G6PC2 transcripts.

Peptide	Location	Sequence	*in vitro* IC_{50} (nmol/l)
$IGRP_{3-11}$	Exon 1	FLHRNGVLI	8195
$IGRP_{23-32}$	Exon 1	YTFLNFMSNV	7251
$IGRP_{62-70}$	Exon 1	VIGDWLNLI	1175
$IGRP_{116-125}$	Exon 3	AMGASCVWYV	787
$IGRP_{117-125}$	Exon 3	MGASCVWYV	1146
$IGRP_{125-134}$	Exon 3	VMVTAALSHT	33673
$IGRP_{130-138}$	Exon 3	ALSHTVCGM	5655
$IGRP_{137-145}$	Exon 3	GMDKFSITL	521
$IGRP_{152-160}$	Exon 4	FLWSVFWLI	255
$IGRP_{155-164}$	Exon 4	SVFWLIQISV	3545
$IGRP_{161-169}$	Exon 4	QISVCISRV	8532
$IGRP_{170-179}$	Exon 4	FIATHFPHQV	1285
Variant B_{28-37}	Neo-junction exon 1/5	FMSNVEDQPL	480
Variant B_{37-46}	Frameshift exon 5	LSLPVCTWLL	3379
Variant B_{38-46}	Frameshift exon 5	SLPVCTWLL	60
Variant C_{66-75}	Neo-junction exon 1/5	WLNLIFKCWL	39420
Variant C_{68-76}	Neo-junction exon 1/5	NLIFKCWLL	2150
Variant C_{69-78}	Neo-junction exon 1/5	LIFKCWLLPA	1708

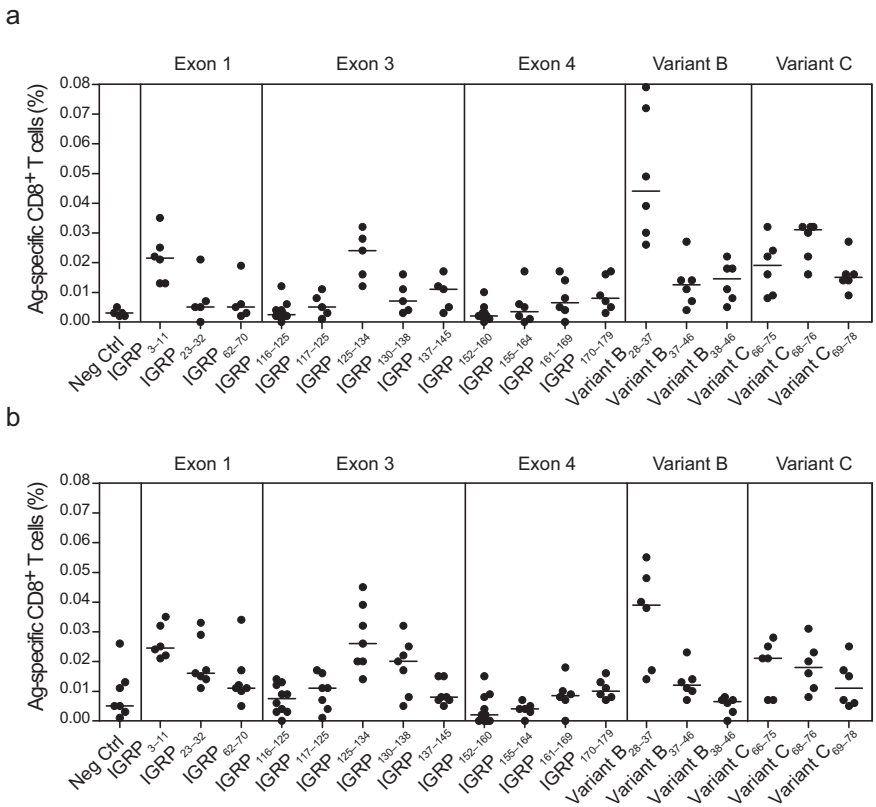

Figure 4. IGRP-specific CD8⁺ T cells in peripheral blood of type 1 diabetes patients and controls. PBMCs of (**a**) recent-onset type 1 diabetes patients and (**b**) healthy individuals were screened for the presence of CD8⁺ T cells recognising IGRP peptides. Peptides are depicted per exon from which they are derived; variants B and C refer to peptides derived from islet-specific isoforms. No significant differences were detected between type 1 diabetes patients and healthy controls using Mann–Whitney *U* tests with Bonferroni correction for multiple testing. Median values are displayed. Ag, antigen; Neg Ctrl, negative control

levels, possibly reflecting the absence of these isoforms from the thymus. Yet, their frequencies were equally high in type 1 diabetes patients and healthy individuals.

DISCUSSION

The thymus plays a central role in tolerance induction by eliminating T cells reactive against peripheral tissue antigens.[1,21] Here, we investigated whether differential splicing between the thymus and pancreatic islets of *G6PC2*, coding for the islet autoantigen IGRP, associated with IGRP autoantibodies and circulating autoreactive CTLs. Expression of *G6PC2* in the thymus and pancreatic islets is similar in quality, yet highly distinct in quantity. In contrast with previous research[16], all conventional splice variants of *G6PC2*, i.e. isoforms using conserved exon–exon boundaries, could be detected in thymic tissues,

even though the expression levels were low compared with pancreatic islets. Despite differences in expression of *G6PC2* between thymus and pancreas, humoral immunity against IGRP splice variants was low in both patients with recent-onset type 1 diabetes and healthy individuals. Relatively high frequencies of $CD8^+$ T cells recognising various IGRP-derived peptides could be detected in peripheral blood of both type 1 diabetes patients and healthy individuals. Thus, while differential expression of *G6PC2* between pancreas and thymus can lead to thymic escape of IGRP-reactive T cells, these autoreactive cells are not associated with disease by definition.

It has been suggested that *G6PC2* is differentially spliced between pancreatic beta cells and the thymus[16]. Here we show that all conventional *G6PC2* isoforms occur in the thymus, including full-length *G6PC2*, albeit in very low frequencies. The only isoforms showing islet-restricted expression in our study were the neo-sequence variants, A, B and C, resulting from alternative splice sites. Since thymic expression of conventional splice variants occurred near the limit of detection, we cannot exclude the possibility that variants A, B and C exist in the thymus. Intriguingly, these particular fragments were associated with the highest rate of islet autoimmunity.

Owing to their putative absence from the thymus, exons 3 and 4 had been proposed to be particularly immunogenic in the pathogenesis of type 1 diabetes because of the lack of central tolerance. Here we show that, although present at low levels, exons 3 and 4 are transcribed in the thymus. Moreover, our findings indicate that this portion of IGRP gives rise to low, rather than high, rates of autoimmunity compared with other IGRP sequences, either because of expression leakage in the thymus or because this region does not contain immunogenic peptide sequences.

In our search for putative IGRP-derived HLA-A2-binding epitopes, we focused on those peptides that are likely to be presented in the thymus despite low thymic expression of *G6PC2*, i.e. peptides with predicted medium- and high-affinity binding to HLA-A2. Recently, however, it has been demonstrated that high frequencies of CD8 T cells recognising low-affinity self-peptides are present in the peripheral blood of type 1 diabetes patients.[7,22] Low-affinity peptides are believed to be presented by mTECs at levels insufficient to ensure proper deletion of autoreactive T cells.[23] Higher expression of tissue antigens in peripheral organs, however, may cause low-affinity peptides to be presented at levels sufficient for T cells to be activated, resulting in immune targeting of self-tissue under conditions of stress or inflammation. As *G6PC2* expression is at least 1000-fold higher in pancreas than in thymus, low-affinity peptides might reach sufficient levels on the cell surface for T cell activation and the subsequent selective destruction of beta cells. Thus, although no differential reactivity against IGRP-derived peptides with high HLA-binding affinity was observed between type 1 diabetes patients and healthy individuals, reactivity against low-affinity IGRP peptides might still distinguish healthy individuals from patients with type 1 diabetes. Further, the immunogenicity of IGRP-derived peptides presented in the context of type 1 diabetes-associated HLA class I molecules other than HLA-A2, such as HLA-A24 or HLA-B39, remains to be determined.

In addition to central tolerance through thymic education, autoreactive T cells can be controlled by other means, such as peripheral tolerance.[24] Extrathymic AIRE-expressing cells have been demonstrated to induce tolerance in a murine IGRP-driven model of autoimmune disease[25] and high-affinity IGRP-specific T cells have been shown to be actively regulated by regulatory T cells in mice.[26] These findings underscore the idea that prevention of autoimmunity goes beyond thymic selection.

Since the discovery of IGRP as an islet autoantigen, its role in the pathogenesis of type 1 diabetes has been controversial. High levels of IGRP-specific $CD8^+$ T cells could be detected in murine models of type 1 diabetes[12], yet, in human type 1 diabetes, the contribution of IGRP to the autoreactive $CD8^+$ T cell population was modest. More recent findings that IGRP knockout mice have delayed onset, but not absence, of islet autoimmunity[27,28] suggest that the notion of IGRP as the driving antigen in the NOD mouse needs revision. The low antibody titres and $CD8^+$ T cell frequencies we have found against IGRP and its isoforms, which are non-discriminative between health and disease, suggest that IGRP does not play a crucial role in type 1 diabetes onset, and further investigation into the relevance of IGRP as an autoantigen in human type 1 diabetes is therefore warranted.

Although we were unable to directly show presentation of IGRP peptides in the thymus or pancreas, in vitro translation of *G6PC2* isoforms indicates that translation into protein indeed takes place. This implies that IGRP splice variants can actually occur in both thymus and pancreas. Thymic presentation of IGRP peptides, however, still appears to be insufficient for complete thymic deletion of IGRP-reactive cells, as demonstrated by their presence in peripheral blood of patients and healthy individuals

In summary, self-reactive T cells can escape thymic selection because of a discrepancy in autoantigen expression between the thymus and pancreatic islets. We propose that the role of the thymus in deleting IGRP-specific T cells is limited, as autoimmunity against IGRP occurs in both type 1 diabetes patients and healthy individuals despite thymic expression of all conventional *G6PC2* isoforms. We disprove the hypothesis that exons 3 and 4 of *G6PC2* are more immunogenic than other IGRP sequences because of differential central and peripheral expression. Furthermore, we conclude that, although self-reactive IGRP-specific T cells escape thymic selection, this does not necessarily cause disease.

ACKNOWLEDGMENTS

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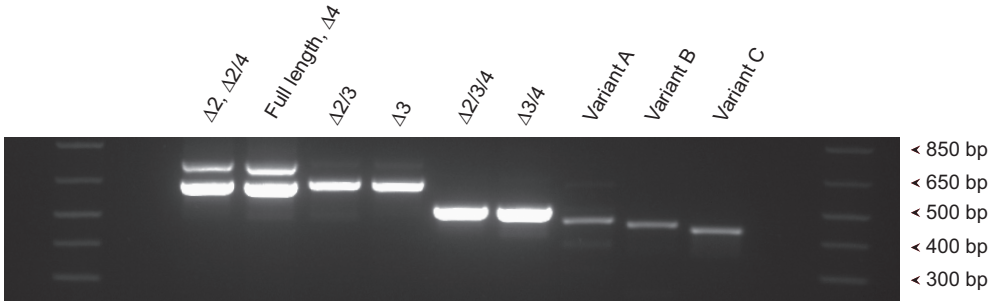
SUPPLEMENTARY DATA

Supplementary Table 1. Primer sequences used for detection of G6PC2 expression.

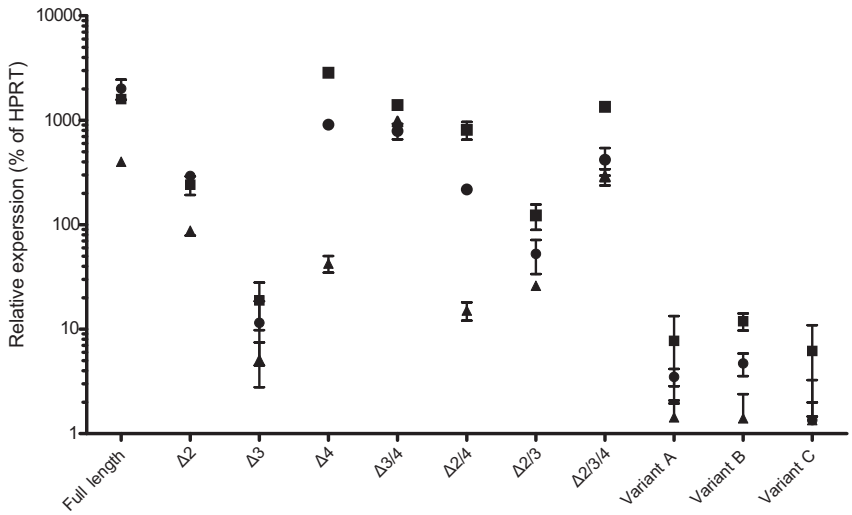
Specificity	Direction	Sequence
Generic isoform amplification	sense	5′-AGAAGGACTACCGAGCTTACTAC-3′
	anti- sense	5′-CAGGTCAATGTTGAGCACCC-3′
Variant A sense	sense	5′-TGGGTAGCAGTCTGGGCA-3′
Variant B sense	sense	5′-GTCCAATGTTGAAGACCAACC-3′
Variant C sense	sense	5′-GGTTAAATCTTATATTTAAATGTTGGC-3′
Splice specific exon 1/3	sense	5′-ATCTTATATTTAAATGGAAGTCCATCTGGC-3′
Splice specific exon 1/4	sense	5′-CTTATATTTAAATGACTGACCTGG-3′
Splice specific exon 1/5	sense	5′-CTTATATTTAAATGGCATGCTGGT-3′
Splice specific exon 2/3	sense	5′-AACAGGTCCAGGAAGTCC-3′
Splice specific exon 2/4	sense	5′-GGTCCAGACTGACCTGGTCATT-3′
Splice specific exon 2/5	sense	5′-AACAGGTCCAGGCATGC-3′
Splice specific common	anti-sense	5′-CTACTGACTCTTCTTTCCGCTTTG-3′

Supplementary Table 2. Primer sequences used for quantitation of *G6PC2* expression by qPCR.

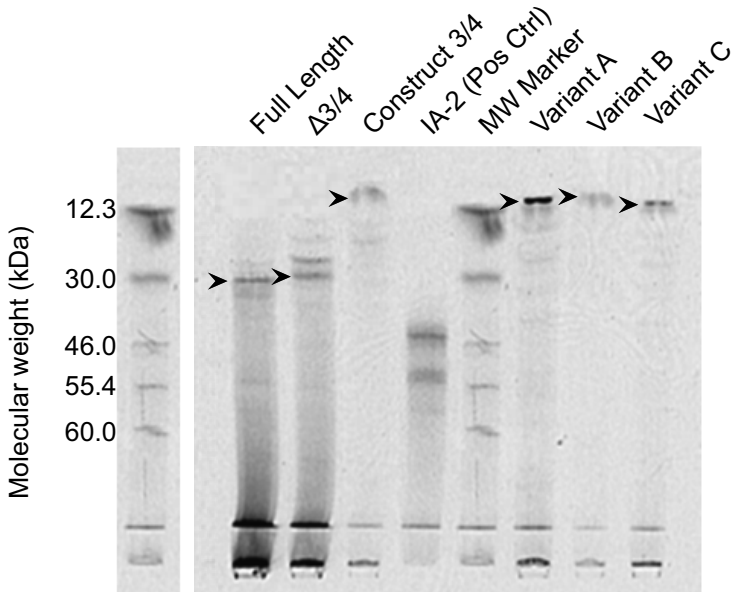
Full length	Sense	5′-AACAGGTCCAGGAAGTCC-3′
	Antisense	5′-CACCAGCATGCCACCAA-3′
Δ2	Sense	5′-ATCTTATATTTAAATGGAAGTCCATCTGGC-3′
	Antisense	5′-CACCAGCATGCCACCAA-3′
Δ3	Sense	5′-GTGAAACAGGTCCAGACTGAC-3′
	Antisense	5′-CACCAGCATGCCACCAA-3′
Δ4	Sense	5′-AACAGGTCCAGGAAGTCC-3′
	Antisense	5′-CACCAGCATGCTGTGCAGA-3′
Δ3/4	Sense	5′-AACAGGTCCAGGCATGC-3′
	Antisense	5′-CAGGTCAATGTTGAGCACCC-3′
Δ2/4	Sense	5′-ATCTTATATTTAAATGGAAGTCCATCTGGC -3′
	Antisense	5′-CACCAGCATGCTGTGCAGA-3′
Δ2/3	Sense	5′-CTTATATTTAAATGACTGACCTGG-3′
	Antisense	5′-CACCAGCATGCCACCAA-3′
Δ2/3/4	Sense	5′-CTTATATTTAAATGGCATGCTGGT-3′
	Antisense	5′-CAGGTCAATGTTGAGCACCC-3′
Variant A	Sense	5′-ATGGATTTCCTTCACAGGAATGGAG-3′
	Antisense	5′-GTGCCCAGACTGCTAC-3′
Variant B	Sense	5′-GTCCAATGTTGAAGACCAACC-3′
	Antisense	5′-CAGGTCAATGTTGAGCACCC-3′
Variant C	Sense	5′-GGTTAAATCTTATATTTAAATGTTGGC-3′
	Antisense	5′-CAGGTCAATGTTGAGCACCC-3′



Supplementary Figure 1. Detection of *G6PC2* isoforms in pancreatic islets using splice specific primers. Intron spanning sense primers were designed to specifically amplify designated *G6PC2* isoforms. A common generic anti-sense primer was used.



Supplementary Figure 2. Expression of *G6PC2* splice variants in pancreatic islets. Relative expression of *G6PC2* was assessed by Q-PCR. Splice variant expression levels differed slightly between islet samples No.1 (circle), No.2 (square) and No.3 (triangle) yet all variants could be detected. Expression of the variant Δ3 and the novel splice variants Variant A, Variant B and Variant C was low compared to other isoforms in all three samples. Error bars indicate 95% C.I.



Supplementary Figure 3. Autoradiography of ^{35}S-labeled in vitro translation fragments after gelelectrophoresis. Arrows indicate predicted protein products

3

SURVIVAL OF AUTOREACTIVE T-LYMPHOCYTES BY MICRORNA-MEDIATED REGULATION OF APOPTOSIS THROUGH TRAIL AND FAS IN TYPE 1 DIABETES

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SUMMARY

Autoreactive $CD8^+$ T cells recognizing autoantigens expressed by pancreatic islets lead to the destruction of insulin-producing beta cells in type 1 diabetes (T1D), but these T cells also occur in healthy subjects. We tested the hypothesis that uncontrolled expansion of diabetogenic T cells in patients occurs, resulting from failure to activate apoptosis. We compared function, transcriptome and epigenetic regulation thereof in relation with fate upon repeated exposure to islet-autoantigen of islet autoreactive T cells from healthy and type 1 diabetic donors with identical islet epitope specificity and HLA-A2 restriction. Patient's T cells proliferated exponentially, whereas those of non-diabetic origin succumbed to cell death. Transcriptome analysis revealed reduced expression of TRAIL, TRAIL-R2, FAS and FASLG (members of the extrinsic apoptosis pathway) in patient-derived compared with healthy donor-derived T cells. This was mirrored by increased expression of microRNAs predicted to regulate these particular genes, namely *miR-98*, *miR-23b* and *miR-590-5p*. Gene-specific targeting by these microRNAs was confirmed using dual-luciferase reporter assays. Finally, transfection of these microRNAs into primary T cells reduced FAS and TRAIL mRNA underscoring their functional relevance. We propose that repression of pro-apoptotic pathways by microRNAs contributes to unrestricted expansion of diabetogenic cytotoxic T cells, implicating microRNA-mediated gene silencing in islet autoimmunity in T1D.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the loss of insulin producing beta cells in the pancreas, leading to impaired glucose homeostasis. Autoreactive cytotoxic T lymphocytes are central to T1D development, as they are responsible for the actual destruction of beta cells. Yet, the processes controlling their undesirable proliferation upon recognition of autoantigens remain poorly understood.[1] Apoptosis, as a physiological means of cell elimination, has a critical role in maintaining immune homeostasis.[2] Several disorders, notably lymphoproliferative disorders and autoimmune diseases, have been associated with disruptions in the mechanisms controlling lymphocyte apoptosis.[3-6] As a general principle, failure to appropriately execute apoptosis in immune cells may lead to uncontrolled survival and expansion of autoreactive cells and thus autoimmune disease.

Apoptosis in mammalian cells can be initiated through a cell-intrinsic and a cell-extrinsic pathway, both leading to the activation of the caspase cascade. The cell-extrinsic pathway depends on the activation of death receptors, such as FAS- or TNF-related apoptosis-inducing ligand (TRAIL) receptors, on the cell surface.[7] The cell-intrinsic pathway involves activation of pro-apoptotic Bcl-2 family members in response to developmental cues and cytotoxic stimuli, such as cytokine deprivation. Which of these two distinct pathways initiates apoptosis in T cells is dependent on the activation status of the cells involved. Naive T cells predominantly commit to apoptosis via the intrinsic Bcl-2-dependent route, whereas apoptosis in activated T cells can be initiated through both the cell-intrinsic and cell-extrinsic pathway.[8,9]

Recently we have shown that beta cell-specific autoreactive $CD4^+$ and $CD8^+$ T cells are present at significantly higher frequency in patients with T1D than in healthy control subjects.[10] Yet, despite this quantitative difference, autoreactivity is not limited to affected individuals, as autoreactive T cells are present in the peripheral blood of healthy subjects as well.[10,11] The presence of autoreactive T cells in unaffected individuals suggests that a qualitative distinction, such as the ability of such cells to proliferate and persist upon activation, might exist. On this basis, we hypothesized that a defining characteristic of autoreactive T cells from T1D patients would be their propensity for unrestricted proliferation upon recognition of cognate islet autoantigen. In contrast, autoreactive cells from healthy controls would be tightly controlled by intrinsic regulatory mechanisms.

To investigate whether proliferative capacities differ between pathogenic and non-pathogenic autoreactive T cells, we compared autoreactive T-cell clones isolated from a T1D patient and a healthy control subject. These clones recognized the same peptide from the putative islet-autoantigen islet-specific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{265–273}$) in the context of HLA-A*02:01 and displayed comparable antigen-specific cytotoxicity *in vitro*. Yet, despite these similarities, the capacity of these clones to expand upon antigenic stimulation differed greatly. While T cells derived from a T1D patient expanded exponentially, those from a healthy individual could only be propagated for a limited number of antigen-specific stimulation cycles. Transcriptome

analysis showed differential gene expression of several pro-apoptotic factors between health and disease, among which are *FAS* (CD95), *FASLG* (CD95L), *TNFSF10* (TRAIL) and *TNFRSF10B* (TRAIL-R2). microRNA (miRNA) profiling revealed that several miRNA predicted to regulate these apoptotic genes, that is, *miR-98*, *miR-590-5p* and *miR-23b*, were simultaneously expressed at higher levels in patient-derived cells. Using dual-luciferase reporters assays, the targeting of Fas and TRAIL pathways by these miRNAs was functionally validated. Furthermore, a reduction of *TRAIL* and *FAS* mRNA following nucleofection of miR-98 into primary T cells was observed, indicating that miRNAs indeed have the propensity to regulate apoptosis through downregulation of cell-surface death receptors and their ligands. Thus, this study identifies a disrupted regulation of Fas and TRAIL by miRNAs as potential mechanism underlying the unrestricted expansion of diabetogenic, autoreactive T cells in autoimmune diabetes.

RESULTS

Autoreactive T lymphocytes from a T1D patient display an increased expansion potential

Previously, we described cloning of autoimmune T cells reactive against amino acid 265–273 of the islet-autoantigen IGRP from peripheral blood of a healthy individual.[12] To our knowledge, this is the first and only islet autoreactive CD8 T-cell clone ever isolated from a healthy donor, putting us in a unique position to compare health with disease. Using fluorochrome-conjugated HLA-A2 tetramer sorting, followed by limiting dilution to give rise to single-cell clones, two independent autoreactive T-cell clones recognizing this same $IGRP_{265–273}$ epitope were isolated from peripheral blood mononuclear cells (PBMCs) of a recent onset T1D patient. All three T-cell clones elicited comparable *in vitro* antigen-specific cytolytic capacity and IFN-γ and granzyme B production upon stimulation with $IGRP_{265–273}$ presented in HLA-A*02:01 and killed HLA-matched primary human beta cells, even though they expressed distinct T-cell receptors (described in more detail in Unger *et al.*,[12] Babad *et al.*[13] and Unger *et al.*[14]). Despite their similarities, $CD8^+$ T lymphocytes derived from T1D blood demonstrated the capacity to expand indefinitely, whereas the healthy donor-derived T cells collapsed after repeated antigen exposure (Figure 1). Thus, patient-derived autoreactive CD8 T lymphocytes appeared to have acquired an enhanced survival capacity leading to increased proliferation, which might be due to a defective apoptosis response to repeated autoantigen exposure.

Reduced expression of apoptotic genes in autoreactive T cells from T1D patients

To determine whether differences in gene expression could explain the enhanced proliferative capacity in diabetogenic T cells, a transcriptome analysis was performed. As each clone has its own growth rate, total RNA was obtained at the end of their respective growth cycle. We focused our analysis on pro- or anti-apoptotic genes with a central role

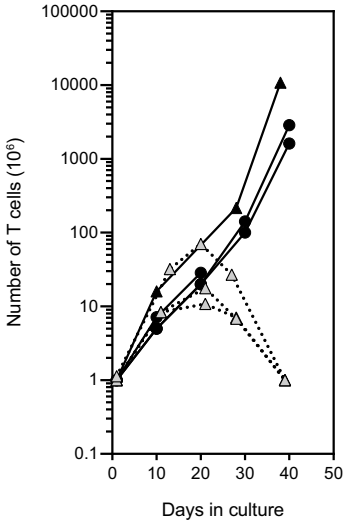

Figure 1. Autoreactive cytotoxic T lymphocytes isolated from T1D patients exhibit increased proliferation rate upon repeated autoantigen exposure. T-cell clone IGRP#7 (black triangle), IGRP#32 (black circle) and healthy individual-derived clone FSB (gray triangle) show qualitative differences in proliferative capacity. Each symbol represents restimulation with cognate antigen $IGRP_{265–273}$. Each line represents a separate passage, showing reproducibility and maintenance of phenotype.

in the intrinsic or extrinsic apoptosis pathway. In addition, members of the caspase family, and genes belonging to the anti-apoptotic inhibitor of apoptosis family were investigated.

Reduced expression of several pro-apoptotic factors from the extrinsic apoptosis pathway was observed in patient T cells, among which are *FAS* and *FASLG*, *TNFSF10*, encoding TRAIL, as well as its receptor *TNFRSF10B* (TRAIL-R2, DR5) (Figure 2a). Protein expression analysis by flow cytometry showed a similarly reduced expression of Fas, FasL, TRAIL and TRAIL-R2 in T1D patient versus healthy individual T cells. Among pro-apoptotic mediators of the intrinsic apoptosis pathway, only the *BAX* showed reduced expression in both patient clones, whereas other genes were not differentially expressed between patients and healthy T cells or did not show consistent expression between the two patient-derived clones (Supplementary Figure 1). Within the inhibitor of apoptosis family, *BIRC5* that encodes the pro-survival factor survivin was upregulated in both patient-derived T-cell clones. Both diabetogenic clones showed reduced expression of the pro-inflammatory caspases *CASP1*, *CASP4* and *CASP5*, as well as the pro-apoptotic caspase *CASP7* compared with healthy individual-derived T cells. Since the Fas/Fas ligand and the TRAIL/TRAIL receptor pathways are known major inducers of T-cell apoptosis, we decided to focus on the mechanisms underlying the discrepant expression of these two major apoptosis pathways between health and disease.

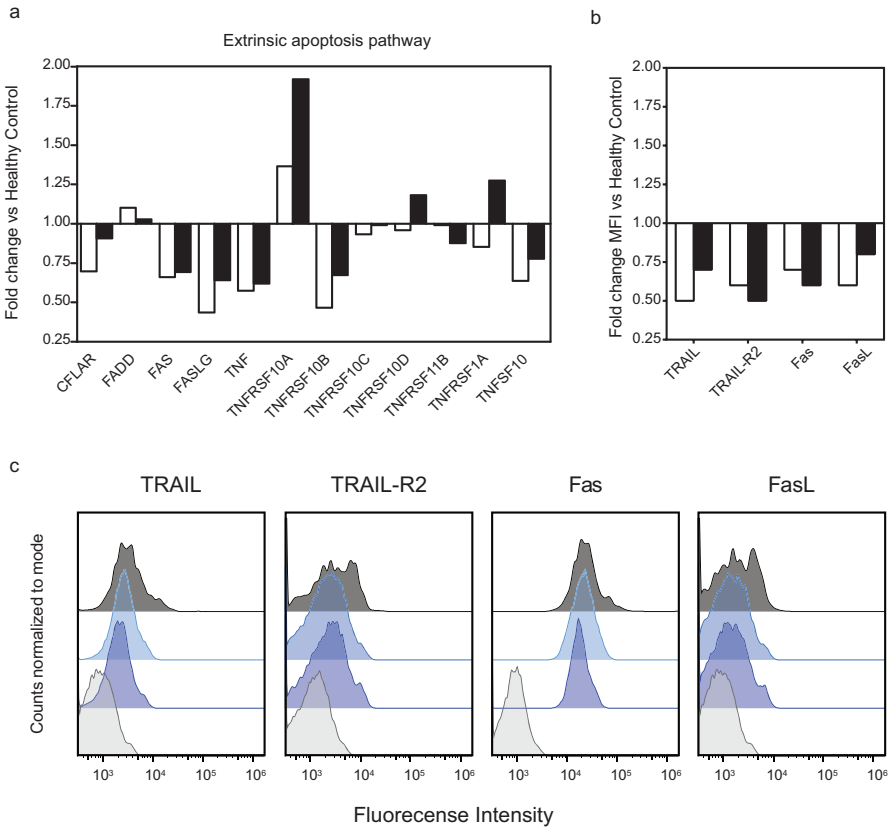

Figure 2. Reduced expression of extrinsic apoptosis pathway factors in diabetogenic cytotoxic T lymphocytes. (a) Gene expression analysis represent patient-derived clones IGRP#7 (white bar) and IGRP#32 (black bar). Values are represented as fold change versus healthy individual clone FSB. (b) Fold change of mean fluorescence intensity (MFI) of patient clones IGRP#7 (white bar) and IGRP#32 (black bar) versus FSB. (c) Fluorescence-activated cell sorting analysis of TRAIL, TRAIL-R2, FAS and FASL on FSB (dark gray), IGRP#7 (blue) and IGRP#32 (purple). Isotype control is displayed in white.

T1D T cells express high levels of miR-98, -590-5p and -23b

Recently it has been shown that death receptor-mediated apoptosis can be regulated by miRNAs, implicating post-transcriptional control in the process of apoptosis regulation.[15] To determine whether miRNAs contributed to the dysregulation of apoptosis in diabetogenic autoreactive T-cell clones, miRNA expression was determined using microarray analysis (Figure 3). Expression profiles differed between patient- and healthy individual-derived autoreactive cells, although the limited sample size did not allow for actual clustering and statistical testing.

To determine whether any differences in miRNA expression could explain the differences found at mRNA level, the 20 miRNAs that showed highest upregulation in both T1D cytotoxic T cells compared with healthy T cells were tested *in silico* for potential regulation

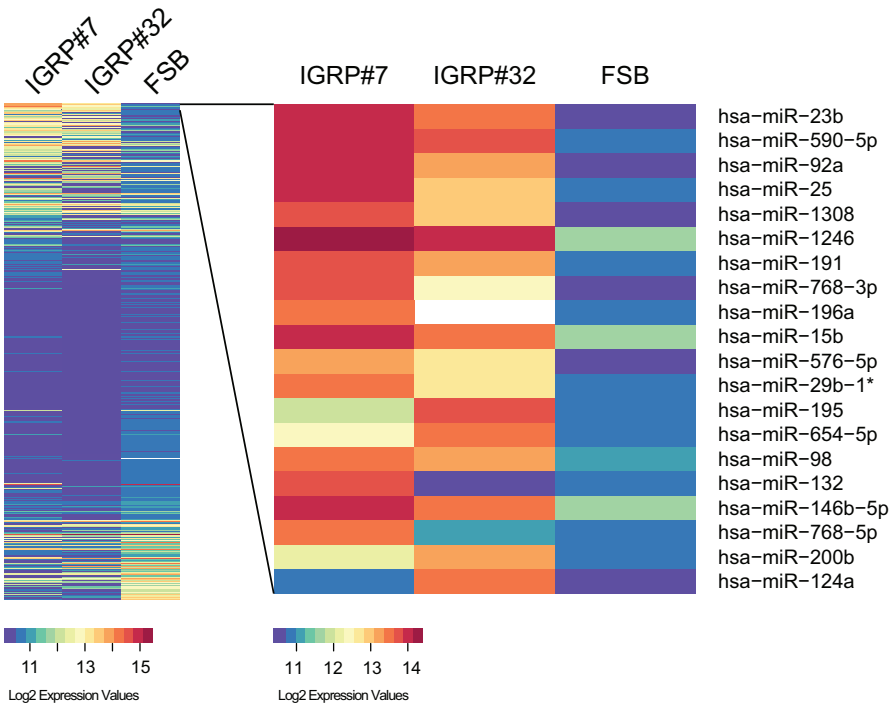

Figure 3. miRNA and mRNA expression in cytotoxic T1D T lymphocytes. Heatmap of miRNA profile showing expression of miRNAs in patient T-cell clone #1 (IGRP#7), #2 (IGRP#32) and healthy control clone (FSB). Ranking was performed as fold change of patient clones versus healthy control.

of *FAS*, *FASL*, *TNFSF10* and *TNFRSF10B* using the online microRNA.org[16] resource. Three of these, that is, miR-98, miR-23b and miR-590-5p, were predicted to target *FAS*, *FASL*, *TNFSF10 or TNFRSF10B* transcripts and were selected for functional follow up (Table 1).

miR-23b, miR-98 and miR-590-5p target members of the Fas and TRAIL pathway

To test whether the predicted miRNA-binding sites in apoptotic genes associated with islet autoimmunity were indeed functional, HEK293T cells were transfected with reporter vectors containing the respective 3′-untranslated region (3′-UTR) of each gene ligated downstream of a luciferase cassette. Control vectors harboring up to two nucleotide mutations at each predicted miRNA target site were created to confirm specificity of miRNA targeting. In the case for TRAIL-R2 targeting by miR-23b, where two predicted binding sites were present, a vector was created where both binding sites were mutated (wild-type and mutated sequences are depicted in Supplementary table 1). Dual-luciferase reporter assays were conducted in the presence of miR-23b, miR-98, miR-590-5p or a negative control miRNA. Transfection of all miRNA lead to specific reduction reporter expression compared with treatment with an *Caenorhabditis elegans*-derived control

Table 1. Predicted miRNA targeting of apoptotic genes

Gene	Predicted miRNA binding
TNFSF10 (TRAIL)	miR-98
TNFRSF10B (TRAIL-R2)	miR-23b,miR-590-5p
FAS	miR-23b,mir-98
FASLG	miR-98,miR-590-5p

miRNA (Figure 4). Specificity of 3'-UTR targeting was validated by calculating the ratio of repression observed in wild-type 3'-UTR constructs over constructs containing miRNA target-mutated 3'-UTRs. Thus, these data indicate that miR-590-5p, miR-98 and miR-23b directly bind to the 3'-UTR Fas, FasL, TRAIL or TRAIL-R2, respectively, thereby inhibiting translation of these pro-apoptotic factors.

Overexpression of miR-98 reduces Fas and TRAIL mRNA expression in primary T cells

To assess the ability of miRNA to regulate pro-apoptotic gene expression in lymphocytes, primary CMV-reactive $CD8^+$ T cells were nucleofected with miR-23b, miR-98 or miR-590. As negative control a *C. elegans* derived, miRNA was used. Forty-eight hours post nucleofection, cells were harvested and mRNA expression of *FAS*, *FASLG TRAIL* and *TRAIL-R2* was analyzed by qPCR. Nucleofection of primary T cells with miR-98 lead to a significant reduction of both *FAS* and *TRAIL* mRNA compared with control miRNA (Figure 5) . In contrast to the reporter assays, *FASLG* mRNA levels were not significantly downregulated upon treatment with miR-98. Nucleofection of miR-23b lead to a reduction of both *TRAIL-R2* and *FAS* mRNA, however efficiency of mRNA reduction varied across experiments and the overall effect only reached statistical significance for *TRAIL-R2*. Downregulation of *TRAIL-R2* mRNA by miR-590-5p was minimal, but comparable to previous findings. miR-590-5p treatment did not result in any downregulation of *FASLG* mRNA, in contrast to the results obtained with previous luciferase reporter assays. Overall, with exception of *FASLG* mRNA, effectiveness of miRNA for their respective targets showed comparable levels of mRNA inhibition in primary T cells compared with previous reporter assay finding.

DISCUSSION

Presence of islet-reactive cytotoxic T lymphocytes is a *conditio sine qua non* for development of T1D. Yet, the mere presence of islet-reactive cells is not sufficient for disease development[10,11] and this observation appears to be generally true, as autoreactive T cells can be routinely detected in blood of healthy subjects.[17] Using a unique series of diabetogenic T-cell clones, we here provide evidence that autoreactive $CD8^+$ T cells from T1D patients differ qualitatively from autoreactive cells of HLA-matched healthy individuals

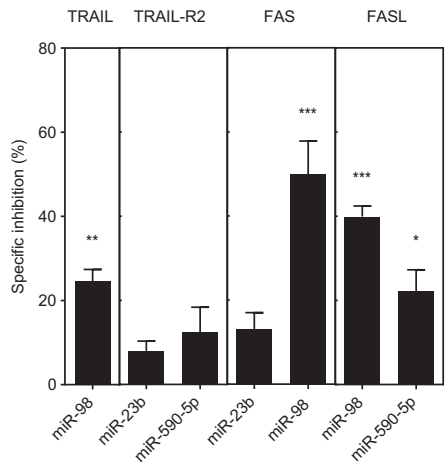

Figure 4. Targeting of extrinisic pathway apoptosis genes by miRNA. The ability of miR-23b, mir-98 and miR-590-5p to target the predicted seed region in the 3′-UTR of *FAS, FASLG, TRAIL* and *TRAIL-R2* was tested with a dual-luciferase reporter constructs containing the wild-type or target site-mutated 3′-UTRs. Specific inhibition was calculated as decrease in luciferase activity in wild-type 3′-UTR compared with target region-mutated 3′-UTR. Each individual experiment was internally normalized against an negative control miRNA (*C. elegans* cel-miR-67). Bars represent mean±s.e.m. Each experiment was performed three times in duplicate. **P*<0.05; ***P*<0.01; ****P*<0.001.

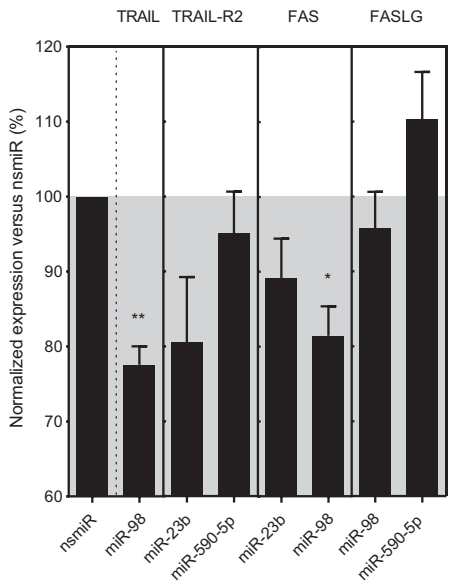

Figure 5. Repression of extrinisic pathway apoptosis genes by miRNA in primary cytotoxic T lymphocytes. For each gene, values were normalized against treatment with negative control miRNA cel-miR-67. Relative mRNA expression was calculated versus housekeeping gene *OAZ1* for all experiments. Bars represent mean±s.e.m. Each experiment was performed at least three times in triplicate. **P*<0.01; ***P*<0.001.

by displaying increased proliferative capacity upon repeated antigen exposure with concomitant reduced expression of the pro-apoptotic *FAS*, *FASLG*, *TRAIL* and *TRAIL-R2* at gene and protein level and increased the expression of miRNAs regulating these factors, that is, miR-23b, miR-98 and miR-590-5p.

One of the hallmarks of autoimmune disorders, and T1D in particular, is the acquisition of defects in the regulatory circuits that control cell proliferation and immune homeostasis. Two well-known pathways involved in immune homeostasis are the Fas/FasL[18] and TRAIL/TRAIL-R pathways,[19] however, their role in T1D appears to be two-fold.[20,21] On one hand, Fas and TRAIL expression by PBMCs is required for control of autoreactive cells,[22] whereas on the other hand, expression of Fas or TRAIL on PBMCs may facilitate beta-cell lysis through increased death receptor:ligand interactions in pancreatic islets.[23,24] It is conceivable that both mechanisms occur simultaneously during autoimmune disease progression. Yet, the immunoregulatory properties of Fas/FasL and TRAIL/TRAIL-R appear to hold greater clinical relevance.[25] This is demonstrated for Fas by increased resistance to Fas-induced apoptosis by PBMC of T1D patients, which would lead to decreased immune regulation and thus increased susceptibility to autoimmunity.[26] Indeed, a favorable response to treatment with high-dose immune suppression followed by autologous hematopoietic stem cell transplantation, correlated with an increase of Fas and Fas ligand expression to levels seen in healthy individuals.[27] Although the role of TRAIL in T1D has not been studied as extensively as Fas, interference with TRAIL receptor signaling correlated with aggravation of autoimmune disease[28] and administration of TRAIL protected against,[29] or alleviated severity[30] of disease in murine models for T1D. These findings suggest that restoration of the apoptosis balance by increasing the expression of Fas and TRAIL pathway members would be beneficial despite possible negative effects on organ damage.

In this study, we focused on the differential expression of the Fas and TRAIL pathway between autoreactive cells from T1D patients and non-affected individuals. Although these two pathways are known regulators of T-cell death, it is likely that other factors contribute to the increased survival capacity of the T cells observed in this study. Indeed, our mRNA profiling experiments point to several other apoptosis regulators that appear differently expressed between autoreactive T cells in health versus disease. For instance, the pro-apoptotic *BAX* was expressed at lower levels in patient-derived autoreactive T cells, whereas survivin, a negative regulator of both Fas- and BAX-mediated apoptosis,[31] was expressed higher in these cells. Further, several members of the caspase family were differentially expressed in patient-derived T cells, among which are the pro-inflammatory caspases *CASP1*, *CASP4* and *CASP5*, as well as pro-apoptotic *CASP7*. Although the individual roles of each of these apoptosis regulators remain to be clarified, these collective findings point to overall anti-apoptotic mRNA expression profile in autoreactive T cells in T1D.

The autoreactive T cells used in this study present a rare opportunity to compare autoreactivity in health and disease. However, as these cells are expanded in vitro we cannot exclude culture artifacts from our analyses, despite the reproducibility of

the observed phenotypes. Significant effort was undertaken to analyze the transcriptome of polyclonal IGRP265-273T cells directly ex vivo, but unfortunately their low frequencies in peripheral blood prevented us to do so in a sufficiently robust and reproducible manner. Further, the fragile nature of autoreactive T cells derived from healthy individuals precluded miRNA overexpression studies, as the procedure of nucleofection resulted in an unacceptable rate of cell death. Therefore, a CMV-specific T-cell clone that expressed FAS and TRAIL at levels comparable to the healthy individual-derived autoreactive T cells were selected to determine the effect of miR-23b, miR-98 and miR-590-5p overexpression in primary T lymphocytes.

Recently, miR-98 has been reported to regulate Fas expression, as well as Fas-mediated apoptosis in a dose-dependent manner in HeLa cells.[15] Furthermore, miR-98 was implicated as mediator of the anti-inflammatory effects of glucocorticoids by suppressing Fas and Fas ligand, among other factors.[32] miR-23b was recently shown to mediate neuronal apoptosis in hypoxia-induced brain damage[33] and overexpression of miR-23b lead to reduced apoptosis in lymphoma cells, whereas inhibition of miR-23b increased cell death.[34] These findings further underscore the general anti-apoptotic potential of these particular miRNAs. Thus far, little is known on the role of miR-590-5p in apoptosis regulation, although miR-590-5p is implicated in acute myeloid leukemia and cervical cancer.[35,36] Interestingly, the seed region of miR-590-5p is identical to the seed region of miR-21, a miRNA overexpressed in a wide variety of malignancies,[37-40] leading to its classification as an 'oncomir'. Overexpression of miR-21 has many consequences, of which the increased resistance to apoptosis is predominant. It can be argued that cancer and autoimmune disease are on opposite sides of the immunological spectrum. Treatment of cancer focuses on activation of adaptive immunity, whereas the therapeutic strategies in autoimmunity target immune regulation. Yet, the finding that two independent but functionally related miRNAs are overexpressed in both cancer and autoimmune disease, with similar phenotypic consequences in both conditions, creates an interesting parallel between autoimmunity and neoplastic disease in which the elucidation of miRNA expression may provide novel therapeutic targets for both conditions.

miRNAs are known essential negative regulators of gene expression during T-cell development and differentiation.[41] A striking example of this is given by miR-155, a miRNA extensively studied for its role in hematopoiesis and lymphocyte functioning. In mice miR-155 deficiency leads to immunodeficiency, likely through dysfunctioning dendritic cells, B and T cells. In contrast, increased expression of miR-155 is linked to tumor development,[42-44] whereas aberrant expression of miR-155 has been observed in several autoimmune diseases including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus.[45] The fact that different levels of expression of a single miRNA can lead to both immune deficiency, autoimmune disease and cancer indicates the potential therapeutic value of understanding the regulatory mechanisms governing miRNA expression. Yet, over the past years insight into miRNA biogenesis has revealed its complexity and to date the exact transcriptional cues that regulate miRNA expression

remain unclear for the majority of miRNAs.[27, 28] miRNA gene control involves various regulatory mechanisms, among which are transactivation and transrepression by nuclear transcription factors. Expression of miRNA can be influenced by extracellular cues and shows tissue- and developmental stage-specific expression.[46] Expression of miR-23b is influenced by extracellular stimuli and inflammatory cues, among which are estrogen, TSH, interleukin (IL)-17 and type I interferons.[47] miR-590-5p expression appears to be affected by IL-3, macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor and is under the control of the lysine methyl transferase SEDT1A (a regulator of cell cycle progression).[35,48] To date, little is known about the molecular pathways regulating mir-98 expression, although v-myc avian myelocytomatosis viral oncogene homolog is implicated in the regulation of the let-7 family, to which miR-98 belongs.[49] Although there is still much to be learned about the spatiotemporal expression of miRNAs, further elucidation of miRNA regulation may aid in identifying a potential common event in the etiogenesis of autoreactive T cells.

miRNAs are inherently pleiotropic and potentially target a wide range of mRNAs. Therefore, functional validation of *in silico* predicted target binding is essential and the biological relevance of miRNAs for complex biological processes such as apoptosis should ideally be tested only in relevant cell types.[50] This is underscored by the discrepancy in miRNA efficacy observed in our study between dual-luciferase reporters assays and primary T cells. Limited availability of autoreactive T cells and technical difficulties manipulating these cells, such as low transfection efficacy and low tolerance for manipulation, impair functional miRNA studies for the most relevant cell types in autoimmunity. Here we used a top-down approach to identify disparities between autoreactive T-cell clones from T1D patients and healthy individuals and identified differential expression of two canonical extrinsic apoptosis pathways and miRNAs regulating them, leading to increased proliferative potential of diabetogenic autoreactive T cells.

Previously, great effort was put into understanding the roles of protein-coding genes in T1D. Our finding that miRNAs can reduce the expression of several key apoptotic molecules in cytotoxic T lymphocytes implicate them as modifiers of disease susceptibility in T1D. Increased expression of miRNAs may act as a biomarker, distinguishing autoreactive T cells in patients from those in healthy subjects. Understanding the mechanisms by which autoreactive T cells overexpress miRNAs leading to increased survival and proliferation upon self-recognition may offer novel targets for therapeutic intervention.

MATERIALS AND METHODS

T cell clones

The procedure for the isolation of healthy controls T-cell clone (FSB) recognizing IGRP have been previously reported.[12] Patient-derived T-cell clones IGRP#7 and IGRP#32 were cloned and characterized, as previously described.[14] All T cells were cultured in Iscove's Modified Dulbecco's Medium with 10% human serum (Sanquin, Leiden, The Netherlands),

supplemented with IL-2 and IL-15. Restimulation was performed in the presence of irradiated, cognate peptide-loaded JY cells and irradiated, pooled third-party PBMCs. All cells used are routinely tested for mycoplasma before freezing. Informed consent was obtained for all material used in this study and was approved by the internal review board of our institution.

mRNA and miRNA profiling

Total RNA, including the miRNA fraction, was purified from cytotoxic T-cell clone derived from T1D patient and healthy donor by the miRNeasy isolation kit (Qiagen, Venlo, The Netherlands). Microarray-based miRNA and whole-genome mRNA expression profiling analysis were performed using Illumina (San Diego, CA, USA) human miRNA and mRNA arrays (Illumina). The miRNA arrays contained ~1200 assay probes corresponding to the all annotated human miRNAs (miRBase, version 12, 2008, The Wellcome Trust Sanger Institute, Cambridgeshire, UK). Total RNA labeling and hybridization was performed using standard conditions according to manufacturer instructions.

Flowcytometry

For flow cytometry analysis, aliquots of 2×10^5 cells were incubated with a cocktail of monoclonal antibodies on ice for 30 min and washed with phosphate-buffered saline supplemented with 0.5% bovine serum albumin. Flow cytometric staining was analyzed on a fluorescence-activated cell sorting Accuri (Becton Dickinson, Breda, The Netherlands). Analyses were performed on Flow Jo 7.6 (Tree Star, Ashland, OR, USA). Antibodies used in this study were FITC-conjugated anti-CD95 (clone DX2, eBiosciences, San Diego, CA, USA), PE-conjugated anti- CD178 (clone NOK-1, Biolegend, San Diego, CA, USA), PE-conjugated anti-CD253 (clone RIK-2, Biolegend) and APC-conjugated anti-CD262 (clone DJR2-4, Biolegend). PerCP-conjugated anti-CD8 (clone SK1) and corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA, USA).

Dual luciferase assays

HEK293T cells were propagated at 37 °C in Dulbecco's Modified Eagle's Medium (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. Cells were regularly passed to maintain exponential growth.

Prior to transfection, cells were plated at 50% density in 24-well format cell culture plates.

Transfection was performed using Lipofectamin 2000 (Life Technologies), 75 µg of reporter plasmid, 15 µg of SV40-RenillaLuciferase plasmid and 50 µg of miRNA mimic (HMI0408, HMI0982 and HMI0814; Sigma-Aldrich, St Louis, MO, USA) or negative control miRNA (MISSION miRNA, Negative Control 2; Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were harvested 48 h post transfection and dual-luciferase activity

was analyzed using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to manufacturer's instructions. Experiments were carried out threefold in triplicates.

T cell nucleofection

CMV reactive primary T cells were nucleofected using Human T Cell Nucleofector Kit (VPA-1002; Lonza, Basel, Switzerland) on the Nucleofector 2 (Lonza). Cells were prepared according to manufacturer instructions with 300 µmol of miRNA mimic. Total RNA from primary CD8 T cells was isolated using Nucleospin miRNA kit (Machery-Nagel, Düren, Germany). cDNA synthesis was carried out using Superscript III (Invitrogen, Bleiswijk, The Netherlands) and oligo-dT primers. Detection of human *FAS*, *FASLG*, *TNFSF10B*, *TNFRSF10* and *OAZ1* was performed using SYBR Green PCR Master Mix (Life Technologies) StepOnePlus Real-Time PCR System (Life Technologies). Primer sequences are depicted in Table 2.

Statistical anlysis

One-way ANOVA tests with correction for multiple testing with Dunnett's test were carried with the statistical package of GraphPad Prism (Graphpad Software, San Diego, CA, USA).

Table 2. RT-PCR primer sequences

FAS	sense	5'-accaaggttctcatgaatctcc-3'
	antisense	5'-tgactccagcaatagtggtgata-3'
FASLG	sense	5'-tggggatgtttcagctcttc-3'
	antisense	5'-gtgtgcatctggctggtaga-3'
TNFRSF10B (TRAIL-R2)	sense	5'-gaagaaagtccttccttacctgaa-3'
	antisense	5'-ccaggtcgttgtgagcttc-3'
TNFSF10 (TRAIL)	sense	5'-cctcagagagtagcagctcaca-3'
	antisense	5'-ggcccagagccttttcat-3'
OAZ1	sense	5'-ggatcctcaatagccactgc-3'
	antisense	5'-tacagcagtggagggagacc-3'

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Author Contributions

V.M.d.J., A.Z and B.O.R. wrote the manuscript; A.R.vd.S created the expression vectors; S.L. performed cell culture experiments; R.v.'t.S. performed the microarray analyses; V.M.d.J and A.Z designed experiments; B.O.R. and B.P.C.K. supervised the project

Conflict of Interest Statement

There are no conflicts of interest to disclose.

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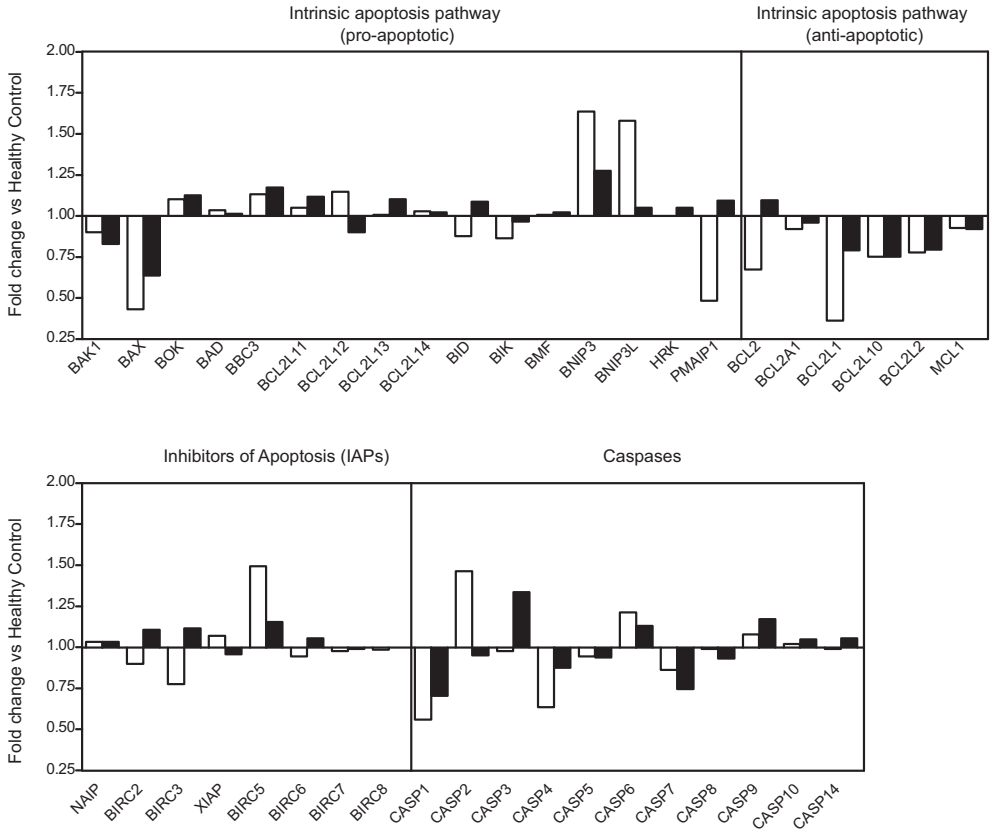
SUPPLEMENTARY DATA



Supplementary Figure 1. Microarray expression analysis of members of the intrinsic apoptosis pathway, Inhibitors of Apoptosis (IAPs) family, and caspase gene family in diabetogenic cytotoxic T lymphocytes. White bars represent patient derived clone IGRP#7, black bars represent patient derived clone IGRP#32. Values are represented as fold change versus healthy individual clone FSB.

Supplementary Table 1. Sequences microRNA binding sites in the 3' UTR of TRAIL, TRAIL-R2, FAS and FASLG. Alterations made to wild-type binding sequences in order to generated mutated constructs are depicted by underscored, capital letters.

Gene	microRNA	Variant	Sequence
TRAIL	miR-98	Wild-type	gactctacctcat
		Mutated	gactcGaGctcat
TRAIL-R2	miR-590-5p	Wild-type	ttttataagctg
		Mutated	ttttaCCagctg
	miR-23b #1	Wild-type	agctgaatgtgat
		Mutated	agctgCaGgtgat
	miR-23b #2	Wild-type	agttatgtgaat
		Mutated	agttaACtgaat
FAS	miR-98	Wild-type	ctctacctcaa
		Mutated	ctctaGctcaa
	miR-23b	Wild-type	taaatgtgaat
		Mutated	taaatCtgaat
FASLG	miR-98	Wild-type	tgctacctcaa
		Mutated	tgctaGctcaa
	miR-590-5p	Wild-type	taataagctaa
		Mutated	taataGgctaa

4

POSTTRANSCRIPTIONAL CONTROL OF CANDIDATE RISK GENES FOR TYPE 1 DIABETES BY RARE GENETIC VARIANTS

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ABSTRACT

The genetic variation causal for predisposition to type 1 diabetes (T1D) remains unidentified for the majority of known T1D risk loci. MicroRNAs function as post-transcriptional gene regulators by targeting microRNA-binding sites in the 3′ untranslated regions (UTR) of mRNA. Genetic variation within the 3′-UTR of T1D-associated genes may contribute to T1D development by altering microRNA-mediated gene regulation. *In silico* analysis of variable sites predicted altered microRNA binding in established T1D loci. Functional implications were assessed for variable sites in the 3′-UTR of T1D candidate risk genes *CTLA4* and *IL10*, both involved in immune regulation. We confirmed that in these genes 3′-UTR variation either disrupted or introduced a microRNA-binding site, affecting the repressive capacity of miR-302a* and miR-523, respectively. Our study points to the potential of 3′-UTR variation to affect T1D pathogenesis by altering post-transcriptional gene regulation by microRNAs.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of pancreatic β cells by T cells.[1] It is appreciated that both environmental and genetic factors contribute to disease predisposition, although the identity of causal factors remains elusive. Identification of the causal genetic factors explaining genetic risk for T1D may vastly improve prediction, prevention and treatment. Genome-wide association studies, assessing millions of single-nucleotide polymorphisms (SNPs) simultaneously in a large number of individuals, have greatly increased the power of genetic association studies and yielded new insights into the genetic architecture of T1D.[2-5] Yet, except for the MHC region, *INS* and *PTPN22*, the causal genetic variations conferring risk for diabetes, and the mechanism on which they act, remains undetermined. Furthermore, the current knowledge on genetic disease predisposition leaves a considerable fraction of disease heritability unexplained.[6] It is not likely that the discovery of new common variants will add to the explanation of heritability, but rather it is expected that rare genetic variations will improve insight in heritability.[7]

Rare SNPs occurring in non-coding regions have the potential to induce phenotypical changes by altering regulatory elements such as promoter sequences[8] or 3′ untranslated regions (UTRs). The latter may influence gene expression levels by interfering with regulation by microRNAs, an important mechanism of post-transcriptional gene regulation.[9,10] Recently it has been shown that SNPs frequently alter accessibility of microRNA-binding sites, which may account for individual differences in gene expression patterns.[11]

We hypothesized that SNPs in 3′-UTRs of T1D-associated candidate risk genes affect T1D pathogenesis by modifying microRNA-binding sites and interfering with gene regulation. SNPs located in 3′-UTRs of T1D-associated genes were selected through dbSNP and T1D Genetics Consortium databases and assessed for their ability to create or disrupt microRNA-binding sites using the PolymiRTS database.[12] Our data show that variation of functionally relevant microRNA-binding elements within the 3′-UTRs of T1D-associated genes can have consequences for regulation of T1D risk genes. This suggests a novel and unexplored mechanism by which rare SNPs can influence disease pathogenesis with relevance for translation of genetic risk and possibly intervention.

RESULTS AND DISCUSSION

Genome-wide association studies have identified 56 genomic regions conferring risk for T1D development and subsequent fine-mapping proposed candidate disease genes for many of the T1D regions.[3] For many T1D loci, more than one gene is implicated, resulting in a total of 268 candidate disease genes. We investigated whether known genetic variation of 3′-UTRs of these T1D risk genes are predicted to alter microRNA binding by cross-reference of known 3′-UTR SNPs of T1D risk genes using the PolymiRTS database.[12] Mature microRNAs exert their repressive function embedded in the multiprotein RNA-induced silencing complex. The structural conformation of targeted mRNA molecules is

known to affect the accessibility of microRNA-target sites to the multiprotein RNA-induced silencing complex and has been shown to potently influence microRNA functionality.[13] Therefore, we performed accessibility analysis of each microRNA-target site to estimate the probability of microRNA-target duplex formation.[14] Accessibility of the predicted microRNA sites was computed as the difference ($\Delta\Delta G$) in free energy gained from the microRNA-target formation (ΔG_{Duplex}) and the energetic cost of disrupting the secondary structure of the target (ΔG_{Open}).

Using the PolymiRTS algorithm, we identified 37 different SNPs within 3′-UTR of T1D candidate disease genes, that could affect microRNA binding (miRSNPs) (Table 1) and 127 in additional genes mapping to the T1D risk loci (Supplementary Table 1). The 37 miRSNPs in T1D candidate disease genes were predicted to affect 50 microRNA-binding sites, of which 28 (56%) involved disruption and 22 (44%) involved introduction of microRNA binding. Accessibility analysis of these sites predicted favorable microRNA–mRNA duplex formation by 13 (26%) miRSNPs using a minimum $\Delta\Delta G$ value of $-7\,kcal\,mol^{-1}$ as cutoff. The remainder (74%) of the microRNA-binding sites predicted by the PolymiRTS algorithm were calculated to have unfavorable binding characteristics, underscoring the need for functional validation of microRNA targets sites procured by *in silico* analysis.

To address the functional impact of polymorphic microRNA sites in T1D risk genes we focused on miRSNPs that were predicted to have functional consequences by both prediction models. As proof of principle for both disruption or induction of microRNA binding we selected miRSNPs in immune-regulatory genes *CTLA4* and *IL10* for functional validation. Both loci have important functions in immune regulation, which is believed to be ineffective in controlling loss of tolerance and immune-mediated beta-cell destruction in T1D. Reporter constructs containing the full length ancestral 3′-UTR sequences of *CTLA4* and *IL10* were generated and were co-transfected with 50 pmol of miR-302a* and miR-523, respectively in HEK293T cells (Figure 1). miR-302a* showed the capacity to inhibit mRNA containing the wild-type 3′-UTR *CTLA4*, whereas the wild-type *IL10* 3′-UTR was not regulated by miR-523. To address the functional impact of polymorphism within the microRNA-target sites we generated the allelic counterparts of the *CTLA4* and *IL10* 3′-UTR by mutating the luciferase reporter constructs at a single-nucleotide position within the microRNA-binding site. Transfection of the mutated 3′-UTR constructs with their respective microRNAs resulted in reduced inhibition of luciferase activity by miR-302a* with the minor allele of the *CTLA4* SNP rs13384548. Conversely, increased gene repression was observed for the minor allele of *IL10* SNP rs6687786. These data demonstrate that, indeed, rare genetic polymorphisms in the regulatory sequences of T1D risk genes have the propensity to alter protein levels by influencing microRNA-mediated gene repression.

T1D diabetes is marked by a large variability of disease occurrence and progression between affected individuals of different ethnicities. As with regular SNPs, the frequency of miRSNPs can differ greatly between different ethnic populations, as demonstrated by the IL10 miRSNP rs6687786, which is considerably more frequent in individuals of African descent than those of Central European descent (6–8% (ref. 15) *vs.* 1.3% in dbSNP, resp.).

Table 1. Polymorphic microRNA binding sites in T1D risk genes. Genes listed by the T1DGC as candidate causal genes were screened for polymorphisms affecting microRNA binding the PolyMIRTS database. Free energy was calculated for the allelic variant containing the predicted microRNA site and the corresponding microRNA. miRSNPs with an estimated ΔΔG of ≤ -7 kcal/mol are displayed in bold. Minor allele frequencies were obtained from dsSNP.

Gene	SNP	Minor allele freq. (%)	Alleles	microRNA	miR site	ΔΔG (kcal/mol)	Consequence
BACH2	rs10455512	21.9	A/G	miR-591	ATGGTCA	-9.73	Disrupt
	rs17513276	3.7	A/G	miR-521	AGTGCGT	n/a	Create
C1QTNF6	rs9622564	0.2	C/T	miR-23a/b	ATGTGAA	-3.40	Create
	rs6000598	n/a	C/A	miR-374	TATTATA	-3.97	Create
				miR-369-3p	TATTATA	-1.92	Create
	rs9622563	n/a	C/T	miR-15a/b miR-16 miR-195 miR-424 miR-497	TGCTGCT	-6.42	Disrupt
	rs5756539	27.1	T/A	miR-15a/b miR-16 miR-195 miR-424 miR-497	TGCTGCT	-6.42	Disrupt
				miR-500	AGGTGCA	n/a	Create
CD226	rs1369896	0.0	A/G	miR-632	CAGACAA	-8.53	Create
				miR-346	GCAGACA	-4.49	Create
				miR-452	GCAAACA	n/a	Disrupt
CD69	rs11052877	34.2	T/C	miR-145	ACTGGAA	1.65	Create
COBL	rs10251388	0.6	G/C	miR-18a/b	GCACCTT	-12.27	Create
				miR-19a/b	TTTGCAC	-5.88	Create
	rs17134126	1.9	G/A	miR-635	GCCCAAG	-3.89	Create
CTLA4	rs13384548	0.1	G/A	miR-302a*	ACGTTTA	-11.17	Disrupt
ERBB3	rs3202538	n/a	G/T	miR-204	AAAGGGA	6.62	Disrupt
				miR-211	AAAGGGA	-2.56	Disrupt
GAB3	rs12395061	6.8	T/C	miR-339	ACAGGGA	-5.42	Create
	rs4431759	46.0	A/G	miR-197	TGGTGAA	-6.23	Create
	rs3813455	4.4	G/C	miR-373* miR-616	TTTTGAG	2.71	Disrupt
IL10	rs6687786	1.3	C/T	miR-523	GCGCGTA	-7.70	Create
IL19	rs2243199	0.4	A/G	miR-654-5p	GCCCACC	-8.41	Disrupt

Table 1. (continued)

Gene	SNP	Minor allele freq. (%)	Alleles	microRNA	miR site	ΔΔG (kcal/mol)	Consequence
	rs2243192	0.0	A/G	miR-26a/b	ACTTGAA	-8.05	Disrupt
	rs2243193	41.2	G/A	miR-617	GAAGTCA	-12.29	Disrupt
	rs1798	18.6	C/G	miR-450	GCAAAAA	n/a	Create
				miR-189	TAGGCAA	-6.99	Create
IL20	rs3024522	n/a	A/G	miR-527	CTTTGCA	-9.23	Disrupt
	rs3024521	1.8	C/T	miR-568	TTATACA	-3.35	Disrupt
				miR-410	TTATATA	-3.13	Create
IL18RAP	rs7559479		A/G	miR-136	AATGGAG	-4.6	Create
PRKCQ	rs4750439	17.0	G/A	miR-564	CGTGCCA	-10.03	Disrupt
	rs11814744	3.4	G/T	miR-452*	AGACTGA	n/a	Disrupt
				miR-622	AGACTGA	-9.94	Disrupt
PTPN22	rs1217412	33.8	T/C	miR-380-3p	TTACATA	n/a	Disrupt
	rs958008	n/a	G/A	miR-576-5p	TTAGAAA	-1.09	Disrupt
RGS1	rs2816308	13.7	G/C	miR-635	CCCAAGA	-2.15	Disrupt
STAT4	rs2280236	n/a	A/T	miR-31	TCTTGCA	-6.04	Create
TAGAP	rs12198374	23.4	A/G	miR-126*	TAATAAT	1.79	Disrupt
TLR8	rs5744087	1.6	C/G	miR-423	CCGAGCA	-18.57	Create
	rs5741889	5.6	A/C	miR-655	TGTATTA	1.05	Disrupt
				miR-641	TGTCTTA	0.82	Disrupt
	rs5744089	0.2	G/A	miR-450	GCAAAAA	1.95	Disrupt
				miR-621	GCTAGCA	-1.91	Disrupt
TNFAIP3	rs12661	n/a	G/T	miR-330	TGCTTTG	-13.47	Disrupt
	rs5029966	0.2	A/T	miR-607	TTTGAAA	-0.26	Create
	rs5029958	1.1	T/C	miR-374	ATTATAA	0.14	Disrupt
				miR-369-3p	TATTATA	2.62	Disrupt
UBASH3A	rs17114952	4.7	C/T	miR-518c*	TCCAGAC	-6.46	Disrupt
				miR-517*	TCTAGAG	-4.78	Create

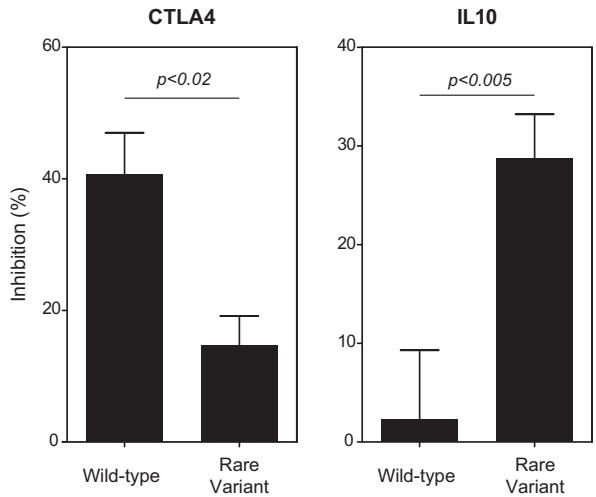

Figure 1. Functional validation of *IL10* and *CTLA4* miRSNPs. Effect of miRSNP in luciferase assay after co-transfection of microRNA and 3'UTR reporter constructs. Full length *CTLA4* and *IL10* 3'UTRs were cloned directly downstream of the firefly luciferase cassette in the pGL3-Control vector (Promega, Madison, WI, USA). Minor alleles of both 3'UTR miRSNPs were obtained by site-directed PCR mutagenesis of the wild-type plasmids. Primer sequences are displayed in **Supplementary Table 2**. Plasmids were sequence verified.
HEK293T cells were maintained in Iscove's Modified Eagles Medium (IMDM) supplemented with 10% fetal calf serum in 5% CO2 atmosphere at 37°C. Cells were transfected at 30-50% confluency in 24-well format with 150ng of either wild-type or rare variant pGL3 plasmid, 25ng pRL-TK (Promega) and 50 pmol of hsa-miR-302a* (Sigma-Aldrich, St. Louis, MO, USA) or hsa-miR-523 (Ambion, Foster City, CA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The ratio of firefly luciferase to renilla luciferase was obtained for each well using Dual Luciferase Assay (Promega). Inhibition was calculated as decrease in firefly to renilla luciferase ratio compared to HEK293T cells transfected with negative control microRNA based on C. elegans microRNA cel-miR-67. Bars represent mean ± SEM. Each experiment was performed at least three times in duplicate. For statistical analysis a Student's T test was used.

As such, miRSNPs may contribute to variation, disease heterogeneity and incidence, and effectiveness of immune intervention therapies targeting T1D between different ethnic populations.[16]

As the phenotypic effects of any miRSNP are ultimately dependent on co-occurrence of both the polymorphic microRNA-binding site and the targeting microRNA itself, further investigation into the dynamics of microRNA expression are required to assess the impact of miRSNPs on disease progression. Our data validate such undertaking by demonstrating that low-frequent 3'-UTR SNPs located in T1D risk genes have the capacity to alter gene expression of potent immune regulators that can contribute to the genetic risk inferred by T1D risk loci.

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SUPPLEMENTARY DATA

Supplementary Table 1. miRSNPs in non-candidate risk gene mapping to T1D risk loci.

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
11p15.5	IGF2AS	n/a	rs17886764	miR-125b	Disrupt
				miR-125a	Disrupt
		38.8	rs10770125	miR-581	Disrupt
		1.1	rs4930042	miR-370	Create
				miR-345	Create
				miR-378	Disrupt
		n/a	rs17880764	miR-586	Disrupt
				miR-448	Disrupt
12q13.2	RAB5B	n/a	rs1050198	miR-154	Disrupt
		n/a	rs12307159	miR-626	Disrupt
				miR-224	Create
		n/a	rs1050200	miR-544	Disrupt
				miR-545	Create
		n/a	rs1050201	miR-370	Create
				miR-661	Disrupt
		n/a	rs1050202	miR-602	Create
				miR-506	Disrupt
		n/a	rs1050203	miR-506	Disrupt
		n/a	rs1050209	miR-448	Disrupt
		n/a	rs7350566	miR-30a-3p	Create
				miR-30e-3p	Create
		n/a	rs1050231	miR-9*	Create
	MYL6	n/a	rs1063598	miR-564	Disrupt
				miR-506	Disrupt
		0.2	rs4847	miR-598	Create
	IL23A	n/a	rs13378047	miR-661	Disrupt
15q25.1	MORF4L1	n/a	rs16970208	miR-532	Create
		n/a	rs1062751	miR-302a*	Disrupt
				miR-517b	Disrupt
				miR-586	Create
				miR-570	Create
		n/a	rs3211410	miR-128a	Create
				miR-128b	Create
				miR-148a	Create
				miR-148b	Create
				miR-152	Create
				miR-586	Disrupt
	RASGRF1	1.6	rs4778732	miR-24	Disrupt
22q12.2	AP1B1	5.0	rs6006095	miR-608	Disrupt
				miR-654	Disrupt
		2.4	rs8140166	miR-130b	Create

Supplementary Table 1. (continued)

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
				miR-301	Create
				miR-130a	Create
				miR-363*	Disrupt
	RFPL1	13.1	rs13053624	miR-425-5p	Create
	NEFH	28.6	rs1061373	miR-661	Create
				miR-637	Create
	NIPSNAP1	29.7	rs7609	miR-577	Disrupt
		n/a	rs13054859	miR-26b	Disrupt
				miR-26a	Disrupt
		n/a	rs459556	miR-153	Disrupt
	NF2	n/a	rs3180505	miR-512-5p	Disrupt
			rs1008515	miR-139	Create
		29.3	rs2530680	miR-328	Disrupt
				miR-504	Create
		30.3	rs1034880	miR-411	Create
				miR-603	Disrupt
				miR-329	Disrupt
		n/a	rs11537543	miR-573	Disrupt
	CABP7	9.2	rs5997508	miR-657	Create
		n/a	rs9614043	miR-515-5p	Disrupt
				miR-519e*	Disrupt
		7.2	rs5752956	miR-617	Create
	ZMAT5	1.1	rs6006230	miR-585	Disrupt
	MTMR3	32.8	rs41171	miR-542-3p	Create
		0.9	rs10212055	miR-566	Disrupt
		33.8	rs12537	miR-181a	Disrupt
				miR-181b	Disrupt
				miR-181c	Disrupt
				miR-181d	Disrupt
		n/a	rs4487184	miR-651	Create
	LIF	26.0	rs737812	miR-617	Create
		0.02	rs11913927	miR-363*	Disrupt
		0.03	rs12160405	miR-324-3p	Disrupt
16p13.13	CIITA	2.4	rs11074940	miR-539	Create
	PRM2	n/a	rs452495	miR-337	Disrupt
				miR-637	Disrupt
	LITAF	n/a	rs13810	miR-7	Create
16q23.1	ZFP1	7.4	rs7199871	miR-512-3p	Disrupt
				miR-520f	Disrupt
		0.0	rs12932318	miR-17-5p	Create
				miR-20a	Create
				miR-106a	Create
				miR-106b	Create
				miR-20b	Create

Supplementary Table 1. (continued)

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
				miR-519d	Create
				miR-93	Create
				miR-302a	Create
				miR-302b	Create
				miR-302c	Create
				miR-302d	Create
				miR-372	Create
				miR-373	Create
				miR-520e	Create
				miR-520a	Create
				miR-526b*	Create
				miR-520b	Create
				miR-520c	Create
				miR-520d	Create
		19.3	rs7206003	miR-496	Disrupt
	BCAR1	7.1	rs11640206	miR-129	Disrupt
				miR-450	Disrupt
19p13.2	ICAM1	25.8	rs281436	miR-373*	Create
				miR-616	Create
		n/a	rs923366	miR-326	Create
				miR-518c*	Create
		25.6	rs281437	miR-31	Create
	ICAM5	n/a	rs2569710	miR-618	Create
		n/a	rs2735443	miR-205	Create
	RAVER1	n/a	rs11539686	miR-299-3p	Disrupt
14q24.1	ZFP36L1	n/a	rs11623420	miR-374	Create
18q22.2	DOK6	n/a	rs1790972	miR-365	Create
22q13.1	SSTR3	1.5	rs8141312	miR-632	Create
				miR-346	Create
21q22.3	TMPRSS3	27.0	rs13047838	miR-30a-3p	Create
				miR-30e-3p	Create
7p15.2	HOXA1	n/a	rs17449017	miR-181a	Disrupt
				miR-181b	Disrupt
				miR-181c	Disrupt
				miR-181d	Disrupt
		n/a	rs17449010	miR-338	Create
		0.4	rs7786554	miR-99a	Disrupt
				miR-100	Disrupt
				miR-99b	Disrupt
	HOXA3	1.1	rs1978133	miR-25	Disrupt
				miR-32	Disrupt
				miR-92	Disrupt
				miR-363	Disrupt
				miR-367	Disrupt

Supplementary Table 1. (continued)

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
				miR-92b	Disrupt
	HOXA4	3.2	rs4722662	miR-197	Disrupt
		3.2	rs4722661	miR-512-5p	Disrupt
	HOXA5	0.8	rs17472021	miR-512-5p	Disrupt
				miR-510	Disrupt
	HOXA7	0.4	rs17472084	miR-551a	Disrupt
				miR-551b	Disrupt
		4.2	rs17500932	miR-187	Create
		n/a	rs17500918	miR-492	Disrupt
	HOXA9	7.0	rs17500987	miR-517*	Disrupt
		36.2	rs7810502	miR-539	Create
12p13.31	CLEC2D	18.3	rs2401388	miR-181a*	Disrupt
		n/a	rs11052488	miR-15a	Disrupt
				miR-16	Disrupt
				miR-15b	Disrupt
				miR-195	Disrupt
				miR-424	Disrupt
				miR-497	Disrupt
		0.1	rs11052491	miR-516-3p	Disrupt
1q32.1	DYRK3	3.0	rs17014165	miR-518c*	Disrupt
3p21.31	FYCO1	12.4	rs2291471	miR-191*	Create
		5.7	rs1994493	miR-554	Create
		9.5	rs1047444	miR-548c	Disrupt
				miR-548a	Create
	CXCR6	46.4	rs2234358	miR-532	Disrupt
	XCR1	n/a	rs7650968	miR-588	Create
				miR-339	Disrupt
	CCR1	6.0	rs3774630	miR-663	Disrupt
				miR-654	Create
	TDGF1	6.9	rs3189859	miR-374	Create
Xp22.2	TMSB4X	n/a	rs11544905	miR-223	Disrupt
19q13.4	CA11	n/a	rs7251936	miR-630	Disrupt
	FUT2	34.0	rs603985	miR-186	Create
		1.2	rs28362840	miR-520g	Disrupt
				miR-520h	Disrupt
				miR-17-5p	Disrupt
				miR-20a	Disrupt
				miR-106a	Disrupt
				miR-106b	Disrupt
				miR-20b	Disrupt
				miR-519d	Disrupt
				miR-518b	Create
				miR-518c	Create
				miR-518d	Create

Supplementary Table 1. (continued)

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
		0.6	rs281376	miR-150	Disrupt
		n/a	rs28746182	miR-492	Disrupt
		32.9	rs2251034	miR-558	Disrupt
		0.3	rs28362843	miR-617	Disrupt
		n/a	rs2638279	miR-637	Create
	FUT1	1.8	rs16982283	miR-518c*	Disrupt
		18.7	rs12611028	miR-517*	Disrupt
		24.7	rs28682322	miR-373*	Create
				miR-616*	Create
Xq28	DKC1	3.5	rs7878787	miR-339	Disrupt
	F8	0.8	rs5986887	miR-542-3p	Create
				miR-34a	Disrupt
				miR-34c	Disrupt
				miR-449	Disrupt
				miR-449b	Disrupt
	FUNDC2	n/a	rs1048795	miR-193a	Disrupt
				miR-193b	Disrupt
		n/a	rs5945283	miR-515-5p	Create
				miR-519e*	Create
	VBP1	n/a	rs17328215	miR-128a	Disrupt
				miR-128b	Disrupt
				miR-27a	Disrupt
				miR-27b	Disrupt
				miR-342	Disrupt
12q13.3	RBMS2	20.7	rs941209	miR-517b	Create
	BAZ2A	n/a	rs11541997	miR-299-3p	Disrupt
		9.4	rs12296335	miR-485-5p	Disrupt
	PTGES3	n/a	rs28413138	miR-489	Disrupt
	STAT6	n/a	rs7316645	miR-630	Create
				miR-626	Disrupt
		n/a	rs11172097	miR-15a	Create
				miR-16	Create
				miR-15b	Create
				miR-195	Create
				miR-424	Create
				miR-497	Create
	R3HDM2	n/a	rs7309842	miR-539	Disrupt
	MARS	n/a	rs1054519	miR-17-5p	Disrupt
				miR-20a	Disrupt
				miR-106a	Disrupt
				miR-106b	Disrupt
				miR-20b	Disrupt
				miR-519d	Disrupt
	DDIT3	n/a	rs1054519	miR-224	Disrupt

Supplementary Table 1. (continued)

Genomic region	Gene	Minor allele frequency (%)	SNP	miRNA	Consequence
				miR-204	Create
				miR-211	Create
	KIF5A	n/a	rs12370421	miR-421	Create
	B4GALNT1	0.4	rs3741418	miR-635	Disrupt
	OS9	n/a	rs10403	miR-605	Disrupt
			rs1050022	miR-373*	Create
				miR-616	Create
		n/a	rs1050038	miR-218	Disrupt
		34.1	rs1050045	miR-639	Create
		n/a	rs1050051	miR-654	Disrupt
	MARCH9	25.6	rs1048691	miR-520a*	Create
				miR-525	Create
21q22.3	PFKL	n/a	rs1057197	miR-542-3p	Disrupt
	C21orf2	1.7	rs9306098	miR-339	Create
2q11.2	AFF3	16.0	rs4851212	miR-595	Create
	LONRF2	19.5	rs2309822	miR-345	Create
		23.3	rs2309821	miR-659	Disrupt
	CHST10	3.8	rs10187094	miR-504	Create
		n/a	rs13419601	miR-491	Create
				miR-625	Create
		3.8	rs6759686	miR-510	Create

Supplementary Table 2. Primer sequences

Primer	Sequence (5′ to 3′)
IL10 3′UTR Cloning F	*AAAAAAAAAA*GCTAGCACAATGAAGATACGAAACTGA
IL10 3′UTR Cloning R	*AAAAAAAAAA*GCTAGCACCAGAACATGATGTGAATAAG
IL10 3′UTR Mutagenesis F	TAGCCGGGCATGGTGGCGCG***T***ACCTGTAATCCCAGCTACTT
IL10 3′UTR Mutagenesis R	AAGTAGCTGGGATTACAGGT***A***CGCGCCACCATGCCCGGCTA
CTLA4 3′UTR Cloning F	*AAAAAAAAAA*GCTAGCAGCAATTTCAGCCTTATTTTATTC
CTLA4 3′UTR Cloning R	*AAAAAAAAAA*GCTAGCAAACGACCACCACAGATTTTTA
CTLA4 3′UTR Mutagenesis F	CTTATATTTACGTATGAGAC***A***TTTATAGCCGAAATG
CTLA4 3′UTR Mutagenesis R	CATTTCGGCTATAAA***T***GTCTCATACGTAAATATAAG

Gene specific region of the cloning primers was elongated with a NheI restriction site (underlined) and a poly-A spacer (italic). Polymorphic site are displayed **bold** and *italic*.

5

VARIATION IN THE *CTLA4* 3'UTR HAS PHENOTYPIC CONSEQUENCES FOR AUTOREACTIVE T-CELLS AND ASSOCIATES WITH GENETIC RISK FOR TYPE 1 DIABETES

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ABSTRACT

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is a protein receptor that downregulates the immune system. *CTLA4* gene variants associate with various autoimmune diseases, including type 1 diabetes. Fine mapping of the genetic risk has shown that the genomic region near *CTLA4* marked by the single-nucleotide polymorphism (SNP) CT60A/G (rs3087243) acts as a susceptibility factor. Yet, the functional basis for the increased susceptibility conferred by rs3087243 remains unclear. We demonstrate that the length of the dinucleotide $(AT)_n$ repeat within the *CTLA4* 3′ untranslated region (3′UTR) strongly associates with the risk of SNP CT60A/G ($P<6.5 \times 10^{-72}$). Genomic $(AT)_n$ repeat length inversely correlated with CTLA4 messenger RNA (mRNA) and protein levels in islet autoreactive T-cell lines. Transfer of a long $(AT)_n$ element into T cells lead to a reduction of mRNA compared to a short $(AT)_n$ element. Thus, this study provides evidence for a role of the *CTLA4* 3′UTR $(AT)_n$ repeat in the increased genetic risk for islet autoimmunity associated with the *CTLA4* locus.

INTRODUCTION

T cells need a co-stimulatory signal in addition to the main antigen-driven signal for full activation.[1] Naïve autoreactive T cells recognizing their target autoantigen may become activated through co-stimulation by CD28 on T cells with CD80 or CD86 on antigen presenting cells.[2,3] Activated Th1 cells orchestrate a cascade of autoimmune responses eventually leading to destruction of self-tissue and autoimmune disease. Cytotoxic T-lymphocyte antigen 4 (CTLA4) is an essential negative regulator of adaptive immunity.[4] CTLA4 has been implicated as an effector molecule of regulatory T cells,[5] and has recently been shown to capture its ligands from opposing cells by process of trans-endocytosis, leading to decreased co-stimulation via CD28 and resulting in negative immune regulation.[6] The relevance of CTLA4 signaling in immune regulation of autoimmune diseases was recently underscored by a clinical trial using Abatacept (CTLA4-Ig) demonstrating temporary preservation of beta-cell function in recent onset type 1 diabetes, although decline in beta-cell function occurred parallel to placebo-treated individuals after 6 months of treatment indicating that in addition to CTLA4 other factors are involved in the dysregulated immune response in type 1 diabetes.[7]

Variations of the *CTLA4* gene region are associated with increased susceptibility to multiple autoimmune diseases, such as type 1 diabetes, [8] celiac disease[9] and rheumatoid arthritis.[10] Despite the identification of the CT60 single-nucleotide polymorphism (SNP; rs3087243) as the principal marker for genetic risk,[8] the mechanism through which polymorphisms of the *CTLA4* locus contribute to autoimmune susceptibility remains undetermined.[11-13]. The *CTLA4* risk haplotype that is marked by the ancestral allele of CT60 has previously been associated with reduced messenger RNA (mRNA) levels of CTLA4 and its soluble isoform sCTLA-4, presumed to be caused by altered gene transcription and splicing.[14,15] Alternatively, reduced mRNA expression might result from altered post-transcriptional control of mRNA stability. Post-transcriptional processing is a regulatory mechanism involved in the protein expression of several immune factors, including tumor-necrosis factor (TNF) and interleukin-1β (IL-1β), which contain regulatory elements in the 3' untranslated region (3'UTR) of their mRNA affecting RNA stability and rate of translation.[16,17] Previously, genetic variation located within 3'UTRs of disease-associated genes has been shown to affect post-transcriptional control and gene expression, thereby potentially affecting disease susceptibility.[18,19]

We investigated whether post-transcriptional regulation of CTLA4 mRNA and protein expression is implicated in autoimmune susceptibility by studying polymorphisms located within the 3'UTR of *CTLA4*. Here we show that a structural variant within the 3'UTR of *CTLA4*, consisting of an $(AT)_n$ dinucleotide repeat, is in strong linkage disequilibrium with the autoimmune risk marker CT60. Long variants of the $(AT)_n$ repeat are associated with reduced CTLA4 mRNA levels in islet autoreactive T cells and transfer of a long $(AT)_n$ element leads to a significant reduction of mRNA in T cells, compared with a short $(AT)_n$ repeat. Thus, variation of the $(AT)_n$ repeat represents an explanation for disease

association of the *CTLA4* locus and implicates altered post-transcriptional regulation of the immunoregulatory CTLA4 as a mechanism contributing to genetic predisposition towards autoimmune disease.

RESULTS & DISCUSSION

Since post-transcriptional control of any gene is largely determined by its 3'UTR, we tested whether the risk SNP CT60 links with variation in the *CTLA4* 3'UTR. Length of an $(AT)_n$ dinucleotide repeat within the 3'UTR of *CTLA4* presents a major source of variation. To determine whether length variation of the *CTLA4* 3'UTR $(AT)_n$ repeat associated with risk for autoimmune disease its correlation with the major risk SNP CT60 was assessed in a cohort of 360 type 1 diabetes patients (Table 1). Analysis showed that CT60 status and $(AT)_n$ length were in strong linkage disequilibrium (D'=1.0, $P<6.5 \times 10^{-72}$). The most common, wild-type, repeat $(AT)_7$ was more frequently observed in individuals carrying the *CTLA4* neutral allele CT60A, whereas alleles containing longer $(AT)_n$ elements were uniquely associated with the CT60G risk haplotype (Supplementary Table 1). Individuals homozygous for CT60A were exclusively $(AT)_7$ homozygous and CT60G homozygosity was strongly related with elongated $(AT)_{variant}$ homozygosity (Supplementary Table 2). Thus, long $(AT)_n$ elements within the 3'UTR of *CTLA4* would directly correlate with increased risk for islet autoimmunity.

To assess the functional consequences of $(AT)_n$ length variation, $(AT)_n$ length was compared with CTLA4 mRNA levels in autoimmune T-cell lines from type 1 diabetes patients and healthy controls reactive against insulin-secretory granules that we had stored in our repository from our studies in the past.[20] These T cells contain islet autoreactivities without bias to certain islet autoantigens. As these T-cell lines had been derived from a series of recent onset type 1 diabetic patients as well as non-diabetic, but age and HLA-matched control subjects, we could assess the relationship between mRNA and protein levels in subjects with susceptible and non-susceptible *CTLA4* associated genetic risk profiles.

The T cells were cultured under IL-2/IL-15 enriched and deprived conditions to mimic physiological conditions that affect mRNA stability [21] and thus expression levels. CTLA4 mRNA levels were significantly lower in T cells with long $(AT)_n$ elements in both patients and healthy individuals, although the low sample size due to the rare nature of islet

Table 1.

CTLA4 AT-repeat	CT60A	CT60G
$(AT)_{wt}$	153	10
$(AT)_{variant}$	0	197

Global D'=1.0 ($p<6.5x10^{-72}$)

Association of $(AT)_n$ length with *CTLA4* risk. SNP CT60A/G was tested in 180 recent-onset type 1 diabetic individuals. Short $(AT)_{wt}$ alleles were associated with the neutral CT60A genotype. Elongated $(AT)_{variant}$ alleles were exclusively associated with the CT60G risk genotype.

autoreactive T-cell lines might pose a possible limitation to this observation (Figure 1a). Furthermore, $(AT)_n$ length correlated with CTLA4 protein expression levels in a series of separately isolated, clonal islet autoreactive T cells with varying $(AT)_n$ lengths in a dose-dependent manner (Figure 1b). To directly test the effect of $(AT)_n$ repeat length on gene expression, Jurkat cells were transduced with a green fluorescent protein (GFP) reporter vector containing the *CTLA4* 3'UTR with either a short $(AT)_7$ or long $(AT)_{28}$ element. T cells transduced with an *CTLA4* 3'UTR containing a long $(AT)_n$ element displayed markedly reduced levels of GFP mRNA, in line with the results obtained from autoreactive T-cell lines and clones (Figure 2). Vector integration was similar between constructs as verified by quantitative reverse transcription PCR (qRT-PCR; data not shown).

Next, we assessed whether this difference in mRNA levels associated with $(AT)_n$ repeat length could be attributed to altered mRNA stability. Jurkat cells were transduced with GFP constructs as described above and treated with the transcriptional inhibitor actinomycin D. Cells were collected at 2, 4, 6 and 8 h after treatment and mRNA expression was measured using quantitative PCR. As observed before, increased reporter mRNA levels were observed for the short $(AT)_7$ levels, however, the rate of mRNA decay

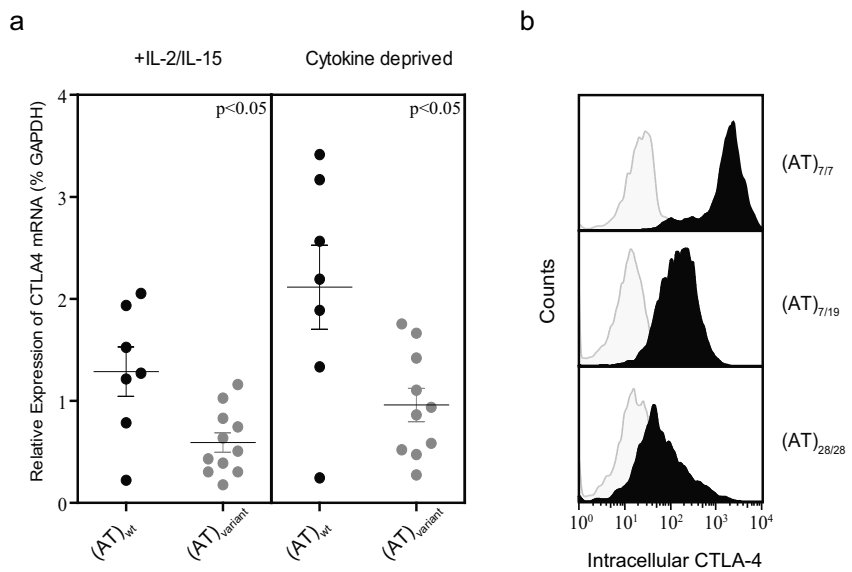

Figure 1. Expansion of the *CTLA4* $(AT)_n$ element correlates with decreased CTLA4 levels in T-lymphocytes. (**a**) qRT-PCR analysis of CTLA4 mRNA in autoreactive T-cell lines cultured in the presence and absence of IL-2 and IL-15. Comparisons between subjects homozygous for the wild-type short repeat ($(AT)_{wt}$) versus subjects homozygous or heterozygous for an elongated element ($(AT)_{variant}$) are shown. For statistical analysis a Mann–Whitney *U*-test was performed. Experiments were performed in triplicates. (**b**) Representative intracellular CTLA4 FACS staining of T-cell clones harboring short ($(AT)_7$), intermediate ($(AT)_{19}$) or long ($(AT)_{28}$) *CTLA4* 3'UTR elements. FACS, fluorescence-activated cell sorting.

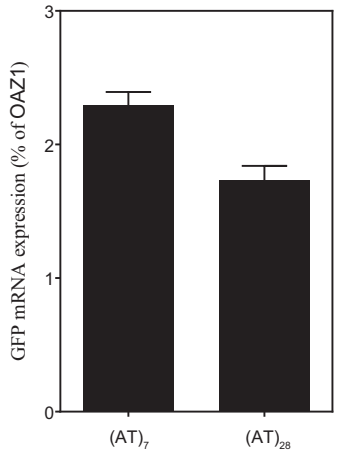

Figure 2. $(AT)_n$ mediates CTLA4 mRNA expression. Jurkat cells were transduced with a vector containing a short ($(AT)_7$) or expanded ($(AT)_{28}$) element ligated downstream of a GFP cassette. qRT-PCR analysis for *GFP* was performed and normalized for the housekeeping gene *OAZ1* in triplicates. Results are shown as mean ± S.E.M.

did not differ between reporter mRNA harboring a short $(AT)_7$ or a long $(AT)_{28}$ element (Supplementary Figure S1).

Variation of the *CTLA4* locus has been implicated in type 1 diabetes, but the genetic variant causal for disease association remains elusive.[22] We reveal a striking linkage disequilibrium between the *CTLA4* risk marker CT60 and $(AT)_n$ repeat length in the *CTLA4* 3'UTR, suggesting involvement of this microsatellite in autoimmune susceptibility. *CTLA4* $(AT)_n$ length affected CTLA4 mRNA and protein levels in islet autoreactive T cells carrying the disease-associated *CTLA4* allele, leading to a reduction in immune regulation in the context of human autoimmune disease. The localization of the $(AT)_n$ element in the regulatory 3'UTR of *CTLA4* implies possible involvement of altered post-transcriptional regulation in disease association of the *CTLA4* locus.

Several other mechanisms by which the *CTLA4* risk allele, marked by CT60G, contributes to risk for autoimmune diseases have been proposed, including differential splicing[15] and altered transcriptional regulation.[14] Differential splicing of CTLA4 mRNA as explanation for the disease association of the *CTLA4* locus remains controversial, and conflicting results have been reported.[23-25] The role of sCTLA-4 in the pathogenesis of type 1 diabetes remains unclear, as circulating sCTLA-4 was only detected in a minority of type 1 diabetes patients by novel isoform-specific antibodies.[26] Further, no direct functional association of promoter polymorphisms and altered *CTLA4* transcription have been found, suggesting that the *CTLA4* risk allele is not functionally related to differences in gene transcription.

Intriguingly, the *CTLA4* $(AT)_n$ repeat is conserved among primates only. This implies that the factors determining genetic risk differ between species despite functional homology of CTLA4. Indeed, the murine *Ctla4* susceptibility allele *(Idd5.1)* is strongly associated with

decreased levels of the ligand-independent isoform of CTLA4 (liCTLA-4),[27,28], a CTLA4 splice variant absent in humans.[15,29] This discrepancy of genetic association of *CTLA4* between species precludes functional genetic studies to define mechanisms of disease association in preclinical animal models.[28]

Collectively, we propose that length variation of the $(AT)_n$ repeat in the 3'UTR is causal for the association of *CTLA4* with autoimmune disease. Using type 1 diabetes as prototype autoimmune disease associated with *CTLA4*, we demonstrated that the *CTLA4* risk allele is functionally related to CTLA4 mRNA expression. We propose that the marker status of CT60 is explained by its exceptionally strong linkage disequilibrium with length variation of the $(AT)_n$ repeat in the *CTLA4* 3'UTR. Individuals carrying risk alleles will have reduced CTLA4 mRNA affecting CTLA4 protein levels and impairing immune regulation. It should be noted, however, that multiple genetic variants may act in concert to modulate disease susceptibility and we cannot exclude that other genetic factors contribute to the genetic risk associated with the *CTLA4* locus. Genetic risk associated with the CTLA4 locus for type 1 diabetes is identical for other autoimmune diseases among which celiac disease and rheumatoid arthritis. Although in this study only type 1 diabetes patients were analyzed, it is likely that our findings can be extrapolated to these autoimmune diseases as well. Our study proposes a novel relationship between genetic susceptibility and variation in post-transcriptional gene regulation, contributing to immune abnormalities that may add to beta-cell destruction.

MATERIAL AND METHODS

Cells lines and clones

Autoreactive T cells against insulin-secretory granules had been isolated from new-onset type 1 diabetes patients (n=10) and healthy HLA and age matched non-diabetic individuals (n=8). Procedures and clinical background reported in detail in ref (20). Jurkat cells were propagated at 37 °C in RPMI-40 supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were regularly passed to maintain exponential growth.

HLA-A2 restricted T cells reactive against insulin, cytomegalovirus and islet-specific glucose-6-phosphatase-related protein were cultured in IMDM supplemented with 10% heat-inactivated human serum, 2 mM glutamine, 100 U ml^{-1} of penicillin, 100 µg ml^{-1} of streptomycin, 20 U ml^{-1} IL-2 and 5 pg ml^{-1} IL-15 at 37 °C in a humidified atmosphere with 5% CO_2. $(AT)_n$ length was determined by sequencing as 7/7 (glucose-6-phosphatase-related protein), 7/19 (cytomegalovirus) and 28/28 (insulin). All cells used are routinely tested for mycoplasma before freezing. Informed consent was obtained for all material used in this study and was approved by the internal review board of our institution.

CTLA4 genotyping

CTLA4 polymorphism (CT60 SNP, rs3087243) analysis was performed on genomic DNA of recent onset type 1 diabetes patients (n=180) using the Taqman SNP genotyping assay

for PCR as supplied by Applied Biosystems (Nieuwerkerk a/d IJssel, the Netherlands). PCR amplification of the $(AT)_n$ element in the 3'UTR of CTLA4 was performed in a total volume of 25 µl, containing 5–10 ng of genomic DNA, 10 pmol of Cy5-conjugated forward primer (5'-CCTTTTATTTCTTAAACAAATGTATGAT-3') and unlabeled reverse primer (5'-CAAAAACATACGTGGCTCTATG-3') at 55 °C. The fragment size was determined using an ABI3730 sequencer. For statistical analysis subjects were divided into two groups: with a short *CTLA4* 3'UTR $(AT)_n$ element (seven repeats) or long $(AT)_n$ element (8 repeats).

qRT-PCR analysis of T cell lines

Total RNA was extracted from autoreactive T-cell lines and primary CD8 T-cell clones using a Nucleospin miRNA kit (Machery-Nagel, Düren, Germany). Complementary DNA synthesis was carried out with Superscript III (Invitrogen, Carlsbad, CA, USA) and oligo-dT primers (Promega, Madison, WI, USA) according to manufacturer instructions. qRT-PCR analysis were performed for *CTLA4* (Sense: 5'-TAGCTTTCTCCTCACAGCTGT-3' Antisense: 5'-TTTTCACATTCTGGCTCTGTT-3') and *GAPDH* (Sense: 5'-TGCACCACCAACTGCTTAGC-3';Antisense: 5'-GCATGGACTGTGGTCATGAG-3') using an iCycler5 with SYBR-green Super-mix (Bio-Rad, Hercules, CA, USA).

CTLA-4 3'UTR cloning and T cell transduction

The *CTLA4* 3'UTR was cloned downstream of the GFP cassette in the lentiviral vector pRRL-GFP using sense primer 5'-AGCAATTTCAGCCTTATTTT-3' and antisense primer 5'-AAACGACCACCACAGATTTTTA-3'. Human DNA isolates were used as template. Constructs and length of $(AT)_n$ region were sequence verified. Total RNA from Jurkat cells was obtained using RNA-Bee (Tel-Test, Friendswood, USA) according to manufacturer's instructions. Complementary DNA synthesis was performed using Superscript III (Invitrogen) and oligo-dT primers (Promega) according to manufacturer instructions. Quantitative RT-PCR was performed with SYBR-Green Master Mix (Life Technologies, Carlsbad, CA, USA) using the StepOnePlus (Life Technologies) system. The following primers were used: GFP (Sense: 5'-GAAGCGCGATCACATGGT-3', Antisense: 5'-CCATGCCGAGAGTGATCC-3'), OAZ1 Sense: 5'-GGATCCTCAATAGCCACTGC-3', Antisense: 5'-TACAGCAGTGGAGGGAGAC-3'). Each experiment was performed at least three times

Fluorescence-activated cell sorting analysis

Ninety-six-well plates were coated with α-CD3 (eBioscience, San Diego, CA, cat #16-0037-85) at a 1:1,000 dilution for 4 h at 37 °C. T cells were incubated on coated plates overnight at 37 °C in full IMDM medium as described above. Before fluorescence-activated cell sorting analysis cells were treated with Golgi-stop (BD Biosciences San Jose, CA, USA; cat. #554724) for 2 h. Cells were fixed and permeabilized using Cytofix/Cytoperm Plus (BD Biosciences, cat. #555028) according to manufacturer protocol. Staining for CTLA4 was

carried out for 30 min on ice using an APC-conjungated α-152 antibody (BD Biosciences, cat. #560938) at a 1:10 dilution. IgG2a-APC (BD Biosciences, cat. #552893) was used as isotype control.

Stastistical analysis

The non-random association between AT length and CT60 was defined by the delta (D') coefficient and was calculated using the UNPHASED software package.[30] As the groups analyzed appeared to have different variances an unequal variances Welch *t*-test was performed to analyze CTLA4 mRNA expression in autoreactive T cells lines. A paired Student's *t*-test was used for comparison of GFP mRNA expression in transduced Jurkat cells. All tests were two-sided.

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CONFLICTS OF INTEREST

Authors declare there are no conflicts of interest to disclose.

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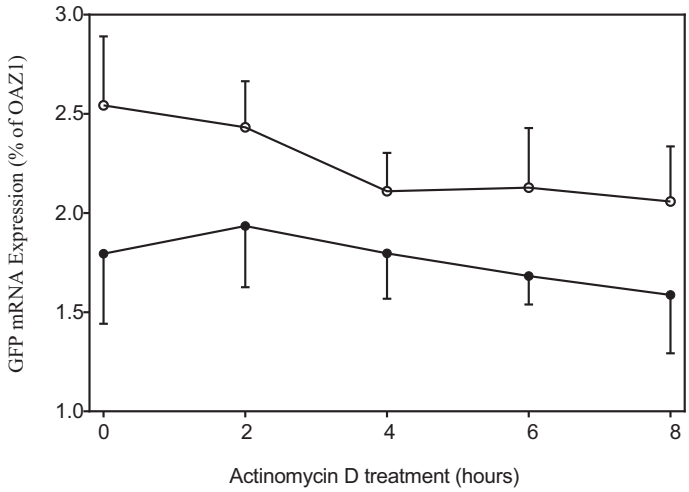
SUPPLEMENTARY DATA



Supplementary Figure 1. *CTLA4* $(AT)_n$ does not affect CTLA-4 mRNA decay. Jurkat cells transduced with a vector containing an $(AT)_7$ (open circles) or $(AT)_{28}$ (black circles) element were treated with Actinomycin D and GFP mRNA was analysed at different time points. Cells were harvested at different time points and qRT-PCR analysis for *GFP* was performed at and normalized for the housekeeping gene *OAZ1* in triplicates. Results are shown as mean ± S.E.M.

Supplementary Table 1. Frequency distribution of $(AT)_n$ alleles

$(AT)_n$	CT60A	CT60G
AT_7	153	10
AT_{11}	0	3
AT_{15}	0	16
AT_{16}	0	90
AT_{17}	0	20
AT_{18}	0	11
AT_{19}	0	9
AT_{20}	0	7
AT_{21}	0	2
AT_{22}	0	3
AT_{23}	0	2
AT_{24}	0	4
AT_{25}	0	8
AT_{26}	0	6
AT_{27}	0	5
$\geq AT_{28}$	0	11

Supplementary Table 2. Association of $(AT)_n$ length with *CTLA4* CT60A/G genotype

CT60 / $(AT)_n$	wt/wt	wt/variant	variant/variant
AA	30	0	0
AG	6	92	0
GG	0	4	58

wt= $(AT)_7$. variant = any $(AT)_n$ >$(AT)_7$

6 GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION AND SUMMARY

Type 1 diabetes is an autoimmune disease that results in the loss of insulin-producing beta cells in the pancreas. The etiology of the disease remains incompletely understood, but both environmental and genetic factors contribute to disease susceptibility. Elucidating the genetic mechanisms involved in disease development will contribute to personalized medicine and is invaluable for the development of novel therapies and a potential cure.

Transcriptional regulation of the islet-autoantigen IGRP is similar between pancreas and thymus and not implicated in the loss of tolerance for IGRP in T1D

In **chapter 2** of this thesis I describe the role of differential splicing of *G6PC2* between pancreas and thymus in the development of islet autoreactivity. *G6PC2* encodes the autoantigen islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a known target of autoreactive CD8 T cell in T1D. Previously, Pugliese et al. suggested that absence of *G6PC2* isoforms containing exon 3 and 4 in the thymus contributes to IGRP autoreactivity.[1] The thymus plays a key role during T cell development as the anatomical site where lymphoid progenitors differentiate and are educated.[2] Through both positive and negative selection a T cell repertoire is formed that can properly interact with antigen presenting cells while avoiding immune responses to the body's own tissue. Potentially autoreactive cells are eliminated in the thymus in a process referred to as central tolerance, which involves presenting tissue-specific autoantigens to developing T cells and the removal of those cells with the potential to react to these autoantigens. That negative selection contributes to the prevention T1D is illustrated by the fact that disease promoting *INS* variant associate with a quantitative reduction of thymic insulin expression, which is thought to impair negative selection of INS reactive T cells and promote islet autoreactivity.[3,4]

In 2006 differential splicing of *G6PC2* between thymus and pancreas was hypothesized to exert an effect on islet-reactivity through incomplete negative selection of IGRP-specific T lymphocytes. We initially set out to quantify the autoreactivity against *G6PC2* isoforms containing exon 3 and 4 in T1D patients, as these isoforms were supposedly lacking from the thymus. However, using *G6PC2* isoform specific primers demonstrated that actually all *G6PC2* isoform that employ conventional splice sites are expressed in the thymus, be it at considerably lower levels than in pancreatic islets. Yet, we did identify three novel splice variants that were only detectable in islet-isolates. As thymic expression of regular *G6PC2* isoforms was already near the lower detection limit of our assays, our inability to detect these in thymic tissue might have resulted from technical limitations, rather than from actual differential expression between pancreas and thymus. Further, these novel islet-specific isoforms employ unconventional splice sites and whether they actually are valid isoforms or erroneous byproducts of normal splicing remains to be determined by proteomic studies and immunohistochemistry. Yet, if proven to be translated into protein

product these isoforms would be an interesting subject for further functional studies, given their exclusivity to islet cells.

Next, we investigated autoreactivity against IGRP isoforms by quantifying IGRP autoantibody levels and frequencies of IGRP-specific, HLA-A2 restricted CD8 T cells in peripheral blood of patients and healthy individuals. Interestingly, T cells reactive against IGRP-derived peptides could be readily detected in the peripheral blood of healthy individuals, suggesting that negative selection of IGRP reactive T lymphocytes is incomplete. Yet, no significant difference could be detected between T1D patients and healthy individuals, implying that the apparent defective negative selection of IGRP-reactive T cells is insufficient development of T1D. In our study we focused on the interaction between IGRP and HLA-A2, the class I molecule that occurs with the highest frequencies among T1D patients.[5] Ideally we would have also been able to analyze T cells recognizing IGRP peptides in the context of other T1D associated HLA molecules, especially HLA-DR and HLA-DQ molecules given their strong genetic linkage to T1D development. Unfortunately, our technical platform currently precludes us from employing any HLA molecule other than HLA-A2. While it is technically possible to produce HLA class II tetramers, *in vitro* generated HLA-class II monomers are highly unstable. Their stability can be improved by covalently linking the peptide of interest to the HLA class II monomer, yet this increases the complexity of their production process and prohibits the high throughput analysis as we have performed here.[6,7] If technological advances would allow a similar approach for class II HLA molecules as we have performed here for HLA-A2, it would be interesting to determine the immunogenicity of IGRP splice variants in the context of the remaining HLA class I and class II molecules to determine their actual impact on the autoimmune response in T1D.

The presence of IGRP-specific T cells in peripheral blood of healthy individuals suggests that incomplete negative selection alone is insufficient for autoimmune disease to develop. IGRP reactive T cells can display potent cytotoxic activity *in vitro* and *in vivo*.[8,9] Therefore, the protection from β-cells lysis in healthy individuals must be effectuated by means other than thymic selection. Potential mechanism involve peripheral suppression of autoreactivity by regulatory T cells (Tregs) or inhibitory mechanisms inherent to autoreactive cells, e.g. increased activation threshold preventing autoreactive T cells from becoming activated, or activation induced cell death (AICD) causing apoptosis upon recognition of an autoantigen.[10,11] The role of the thymus in self-tolerance induction of CD4 T cells was recently shown to be dependent on the autoantigen involved.[12] Although our study was not designed to answer this question, it appears that distinct methods of self-tolerance induction exist for CD8 T lymphocytes as well and the role of the thymus in the prevention autoreactivity against IGRP is limited.

Since its discovery as an islet-autoantigen IGRP has been regarded as one of the driving antigens for disease development and progression in mice.[13-15] However, its exact role in the pathophysiology of human T1D remained controversial as the frequency of IGRP-specific CD8 T cells in the peripheral blood of T1D patients is modest compared that

to murine models.[16,17] In our study antibody titers and CD8+ T cell frequencies against IGRP and its isoforms were low and non-discriminating between health and disease. This suggests against the role of disease-driving antigen for IGRP in human T1D. Still, previous studies from our laboratory showed that IGRP-specific CD8 cells are present in human insulitic lesions and adoptive transfer of human IGRP-specific CD8 T cells into HLA-A2 transgenic mice provokes insulitis and β-cell destruction.[8,18] Thus, while IGRP does not appear to be a driving autoantigen in human autoimmune diabetes, IGRP autoreactivity seems to contribute to the etiology of T1D and clarifying its exact role it would be worthwhile.

Concluding, the difference of G*6PC2* expression between pancreas and thymus is not so much quantitative as it is quantitative. Further, it appears the role of the thymus in tolerance induction against IGRP is limited at most and the effect of reduced thymic *G6PC2* expression on the occurrence of autoimmune disease appears negligible.

Posttranscriptional control of pro-apoptotic genes by miRNA contributes to apoptosis resistance in autoreactive T cells in T1D

In T1D autoreactive CD8+ T-cells destroy insulin-producing β-cells that display their cognate autoantigen on the cell surface. Yet, as described in chapter 2 islet-reactive T-cells can be readily detected in the peripheral blood of healthy individuals. A potential difference between health and disease may be the capacity of peripheral regulatory T cells to dampen autoimmune responses or prevent them from even happening.Reduced Treg activity has been described for several autoimmune diseases, including T1D.[19]and, increased resistance for regulation by autoreactive T effector cells has been suggested as well.[20] In chapter 3 of this thesis we compared two autoreactive T cell clones isolated from a T1D patient with a T cell clone isolated from a healthy individual, all recognizing the same peptide:HLA complex and displaying comparable *in vitro* cytotoxicity. We observed that the healthy individual's autoreactive T cells could be stimulated for a finite number of times before collapsing, a characteristic not observed in autoreactive T cells obtained from a T1D patient. Therefore, we tested the hypothesis that in T1D uncontrolled expansion of diabetogenic T cells occurs as a result of failure to activate apoptosis upon repeated antigen exposure.

Transcriptome analysis of the T cells clones revealed reduced expression of TRAIL, TRAIL-R2, FAS and FASLG, members of the extrinsic apoptosis pathway, in patient-derived compared to healthy-donor-derived T cells. This was mirrored by increased expression of microRNAs (miRNAs) predicted to regulate these particular genes, namely miR-98, miR-23b and miR-590-5p. Gene specific targeting by these microRNAs was confirmed using dual-luciferase reporter assays. Finally, transfection of these microRNAs into primary T-cells reduced FAS and TRAIL mRNA expression, underscoring the functional relevance of these microRNAs in effectuating resistance to apoptosis of autoreactive T cells. Thus, we showed that the differences in proliferative capacity between autoreactive T cells

from health and disease associates with altered in expression of pro-apoptotic genes and the post-transcriptional factors regulating them.

To definitively prove that the increased proliferative capacity of autoreactive T cells from T1D patients stems from altered expression of pro-apoptotic factors and the microRNAs governing them, we set out to reverse the observed phenotypes via molecular intervention. Primary T lymphocytes are notoriously difficult to transfect, but we show that nucleofection is a viable way of introducing miRNA into primary autoreactive T cells. Unfortunately, employing this method on T-cells derived from non-diabetic donors caused cell death in the majority of cells within 24 hours after treatment. This timeframe fell short of the minimal time needed to observe an effect of miRNA treatment, as a resting/recovery phase for the cells and the time required for the miRNA to assert its effect needs to be taken into account. Another difficulty with our initial approach was that the estimated half-life for miRNAs is approximately 5 days.[21] The proliferative capacity of a T cell clone can only be reliably tested at the end of its respective restimulation cycle, which is around 10 days for the autoreactive T cell clones described here. To deal with these limitations, we opted to use virus-specific T cells that express high levels of FAS and TRAIL and are relatively resistant to the negative effects of nucleofection and measure the effect of our treatment with quantitative PCR analysis of FAS and TRAIL mRNA. By doing so we were able to provide evidence that overexpression of miR-23b, miR-98 and miR-590-5p indeed leads to reduced expression of members of the FAS and TRAIL pathways and thereby may contribute to increased survival of autoreactive T cells.

Our observations and transcriptome comparisons in this study involved a single $IGRP_{265-273}$-reactive T cell clone derived from a healthy individual and two $IGRP_{265-273}$-reactive T cell clones from a T1D patient. Autoreactive CD8+ T cell clones are extremely difficult to produce and maintain in culture without loss of antigen specificity, especially autoreactive T cells derived from healthy individuals. The clones used in this study represent the majority of IGRP-specific CD8 T cell clones available worldwide, and although the limited number of samples poses a potential sampling bias, here we have been able to reproducibly investigate the transcriptomes of autoreactive cells that were actually primed *in vivo*, and thus likely involved in β-cell destruction, with their non-pathogenic counterparts from healthy individuals. Naturally our study would have been strengthened if more clones could have been examined, particularly from the healthy individuals. We have aimed to obtain and compare IGRP specific cells directly after isolation from the blood of T1D patients and healthy individuals using a FACS-based approach in an attempt to increase the number of T cell clones for our study. Due to the low numbers of circulating autoreactive cells of a given specificity in both T1D patients and healthy individuals, using this approach we were unable to robustly test mRNA and miRNA expression in T cells with a single specificity using this approach. Yet, by comparing pooled autoreactive cells with varying specificities from recent onset T1D patients to pooled virus-reactive cells from the same individuals we were able to show a significant difference in FAS and TRAIL expression between autoreactive T cells and virus-specific T cells within a single individual,

which lends support to the notion that autoreactive T cells have impaired expression of surface death receptors and a subsequent increased resistance to apoptosis induction (unpublished data). Further, given the promising results from these preliminary data I am convinced that this approach stands to be an excellent alternative to the laborious cell-culture and may offer new opportunities to analyse autoreactive T cells directly ex-vivo in the near future.

Thus, repression of pro-apoptotic pathways by miRNAs contributes to unrestricted expansion of diabetogenic cytotoxic T-cells, implicating miRNA-mediated gene silencing in islet autoimmunity in T1D. Further analysis of the miRNA transcriptome of autoreactive cells might provide novel therapeutic options and elucidation of the mechanisms that govern miRNA expression may result in the identification of (environmental) triggers involved in T1D development.

Rare variants alter post-transciptional regulation of T1D risk genes

In chapter 4 of this thesis we investigated the possible interaction between rare genetic variants and functionality of miRNAs, which play an important role in the post-transcriptional regulation of gene expression. Genome wide association studies (GWAs) have greatly increased our knowledge on disease-associated genomic regions. However, even when taking all verified disease-associated gene variations into account we still cannot completely explain the heritability of T1D. GWAs are designed for the detection of common single nucleotide polymorphisms (SNPs). Rare SNPs and structural variations such as insertions, deletions and repeats are not analyzed with current GWAs and it is therefore hypothesized that the 'missing heritability' of complex diseases such as T1D can be explained by these types of genetic variations.

The magnitude of the missing heritability of T1D is subject to debate, with estimates for the unexplained heritability ranging from 0% to 40%.[22-25] Regardless of the size of the unexplained heritability, consensus is that common genetic risk variants are unlikely further our understanding and that future research should focus on structural and rare variants.[26]

In our proof-of-concept study we have investigated rare polymorphisms in T1D risk genes with the propensity to influence post-transcriptional gene control by affecting miRNA function. miRNAs in general are pleiotropic, with each miRNA regulating up to several hundreds of protein-coding mRNAs. In our study we limited our investigation to genes with known association for T1D susceptibility and investigated whether they were predicted to be under regulatory control by miRNAs. Using *in silico* modeling we identified miRNAs that could potentially interact with mRNA of T1D genes. With gene-targeting models we validated these predictions and demonstrated *in vitro* that rare SNPs, miRSNPs, have the capacity to modulate post-transcriptional gene control by instilling or abrogating miRNA-mediated gene silencing, a novel mechanism through which rare polymorphisms can affect T1D associated gene pathways. Although this study is a proof-of-concept rather

than a comprehensive analysis, we already describe several functionally active miRSNPs in T1D associated gene loci. Currently little is known about the biological importance of the miRNA identified in this study, i.e. miR-302a* and miR-523, and the expression patterns of these miRNA. However, knowledge on both miRNA species and genetic polymorphisms is rapidly increasing and the association between environemtal cues and miRNA expression is becoming more clear.[27] Although further research is required to establish the actual impact of miRSNPs on development and heritability of autoimmune diseases, this study provides a potential mechanism by which environmental factors can interact with genetic susceptibility factors in the development of T1D.

The CTLA 3' UTR $(AT)_n$ microsatellite is causal for the association of the CTLA4 locus with genetic susceptibility for T1D

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a surface molecule present on activated T cells that inhibits the T cell receptor signalling upon binding to its ligands CD80 and CD86. It has been hypothesized that inherited variations in the *CTLA4* gene can increase T cell autoreactivity and thereby play a role in autoimmune diseases such as T1D. Indeed, the *CTLA4* locus was among the first genomic regions to be associated with susceptibility for T1D. Yet, the genetic variant causal for the actual association, as well as the molecular mechanisms associated with increased disease susceptibility have long remained unknown. Recent fine mapping studies have shown that the region marked by the single-nucleotide polymorphism CT60 (rs3087243) downstream of the *CTLA4* 3'UTR acts as a susceptibility factor.[28,29] This CT60 polymorphism maps to a non-coding region and the mechanisms through which it would influence CTLA4 expression remains elusive. In chapter 5 of this thesis we provide evidence that a structural genomic variant, i.e. the $(AT)_n$ microsatellite located within the 3' untranslated region (UTR) of *CTLA4,* is causal for the genetic association of *CTLA4* with T1D susceptibility. First, we investigated the association of the *CTLA4* $(AT)_n$ microsatellite with the T1D risk marker CT60 . Analysis showed that CT60 status and $(AT)_n$ length were in extremely strong linkage and that *CTLA4* alleles containing longer $(AT)_n$ elements are uniquely associated the CT60G risk haplotype. Conversely, the protective CT60A haplotype was observed more frequently in association with wild-type, short, $(AT)_n$ elements. Thus, long $(AT)_n$ elements within the 3'UTR of *CTLA4* would directly correlate with increased risk for islet autoimmunity. In autoreactive T cell lines increased microsatellite length associated with a significant decrease in CTLA4 mRNA expression and direct transfer of a long $(AT)_n$ microsatellite resulted in decreased reporter mRNA expression compared to the transfer of a short $(AT)_n$ element.

It should be noted that the number of T cell lines studied we have studied here, i.e. 10 patient T cell lines and 8 control lines, is low and represents a potential limitation to our findings. Yet, it should also be appreciated that the cell lines used here are extremely rare and generation of additional cell-lines is technically highly demanding. Furthermore, the fact that a significant difference between the lines when stratified on $(AT)_n$ genotype

was still detected, despite the heterogeneous composition of the cell lines, indicates robustness of the association between $(AT)_n$ genotype and CTLA-4 expression.

In order to assess the effect of the $(AT)_n$ length on CTLA4 expression we transferred the 3'UTR of CTLA4 variants with different $(AT)_n$ lengths into a GFP reporter constructs. Sequencing analysis excluded any variation other than the $(AT)_n$ element between the generated constructs. Upon transduction of the constructs into a immortalized lymphocyte cell line we observed a significant difference on steady state GFP mRNA levels, with the construct containing a long $(AT)_n$ element showing decreased levels of reporter mRNA. The use of a long-standing immortalized cell line instead of actual T lymphocytes introduces the possibility of culture artefacts or aberrant outcomes due to differences in intracellular mechanisms, with the addition of potential genomic instability due to the process of transduction. Yet, as our results were consistent over two separate rounds of transduction, performed with separately produced virus batches, we are confident these confounders were kept to a minimum and our results reflect the actual influence of the $(AT)_n$ repeat on gene expression.

The location of the microsatellite in the 3' UTR, known for its role in post-transcriptional gene regulation, suggests that the mechanisms underlying the observed differences in mRNA expression involve altered post-transcriptional control. In our study we did not observe any significant effect of the $(AT)_n$ repeat on mRNA decay rate. While this suggests that the $(AT)_n$ repeat does not affect mRNA stability it remains possible that transcription-dependent factors have acted as rate-limiting factors in our experiment. Proteins with a very short half-life, or a RNA-silencing moieties that are consumed as a result of their actions would be equally affected by the actinomycin D treatment used to block the transcription of our GFP reporter. The actinomycin D treatment may thereby have obscured any mRNA decline that would have normally occurred *in vivo* between short and long $(AT)_n$ containing mRNA. Alternatively, the difference in mRNA levels may be the result of different transcriptional regulation, and not altered post-transcriptional control. It is known that the location of a genomic element does not necessarily correspond with its function and that genomic interactions can occur between regions that are up to hundreds of kilobases apart.[30] Therefore, while its localization suggests that the $(AT)_n$ microsattelite affects post-transcriptional regulation, possibly via short-lived regulatory molecules, our current data do not allow us to exclude long-range gene interactions or differential binding of chromosomal moieties as factors in the observed difference in *CTLA4* mRNA expression. Still, the extremely strong association between the CT60 risk variant and long $(AT)_n$ alleles, combined with the direct effect of the $(AT)_n$ repeat on mRNA expression suggests a causal role for the $(AT)_n$ microsatellite in the genetic association of the CTLA4 region with T1D susceptibility.

FUTURE PERSPECTIVES

The last two decades have provided (bio)medical scientists with a wealth of information on the genetics of complex diseases such as T1D. Understanding exactly how gene variants influence disease susceptibility and determining the interplay between environment, genetics and immunology have proven to be the next hurdles to overcome. Achieving this goal may enable us to predict disease progression, design novel treatment methods and improve patients categorization in order to provide actual personalized medicine. The contributions of this thesis are summarized in Figure 1. In short, we reposition the proposed alternative splicing of *G6PC2* between thymus and pancreas, discuss the role of the thymus in tolerance towards the putative islet-autoantigen IGRP and argue the validity of IGRP as an critical islet-antigen in T1D. Further we show that a structural genetic variant located with the 3' UTR of *CTLA4* associates with risk for T1D development and influences expression of the immune regulator CTLA-4. In addition, we show that rare genetic variants can influence microRNA mediated post-transcriptional control, identifying a novel mechanism through which genetic predisposition might interact with environmental trigger to influence (auto)immunity and contribute to familial aggregation of T1D. Finally, we show that pivotal apoptosis pathways are affected in autoreactive T lymphocytes of T1D patients and that microRNA play a vital role in this, again indicating the importance of understanding of gene-gene and gene-environment interactions in the development of T1D. Yet, our work only covers a small part of the unknown and many aspects of the functional genetics of T1D remain to be addressed. As mentioned before,

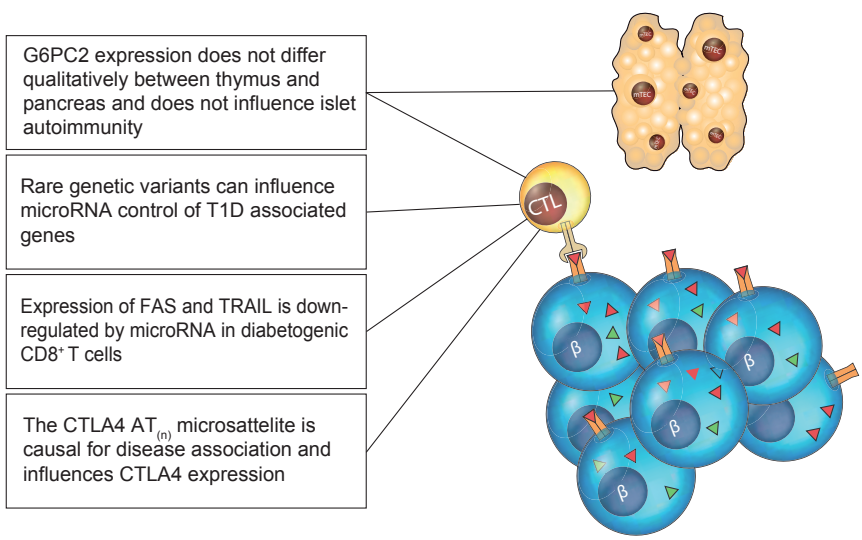


Figure 1. The immune reaction in Type 1 Diabetes – an update. Adapted from B.O. Roep, Nature, 2007[37]

despite their valuable contribution thus far, larger GWAs studies are unlikely to aid this cause further, as potential new risk SNPs are expected to carry a very modest impact on disease susceptibility at best. With current technological advances and the advent of large sequencing centers, whole-genome sequencing, a technique that charts the complete genome of a single individual, has come within reach for academic researchers. Using whole-genome sequencing, future research will be able to overcome the limitations of GWAs by assessing al genetic variation within a single individual at once. At the same time this approach will generate an unparalleled amount of complex data. Data analysis, processing and statistics will most likely prove to be a bottleneck and new strategies for data-management will be required. From the cell-biological perspective of T1D research, optimization of single cell techniques that will allow for direct isolation and investigation of autoreactive T cells directly isolated from peripheral blood can eliminate cell culture artifacts and provide 'clean' data regarding the make-up of diabetogenic T cells and what distinguishes them from their non-diabetogenic counterparts. Further, direct isolations will allow for high-throughput analysis and remove the problem of limited sample availability currently complicating research. Combined with improvements in big data analysis, this approach may prove invaluable in identifying molecular pathway involved in, or even preceding, β cell destruction.

THERAPY

The cure and complete prevention of disease occurrence is the ultimate goal in medicine, but is also be the hardest goal to achieve. Fortunately, treatment modalities are constantly improving, allowing for better patient care in the interim. The future holds several promising outlooks for management of T1D, among which the artificial pancreas[31], stem-cell derived (neo) β cells[32] and promising immunotherapies.[33-35] Recent myeloablative therapies with subsequent autologous stem cell transplantation that resulted in reduced, and in some cases very long-standing, insulin dependency demonstrate the power of immunotherapies and indicate that reprogramming the immune system may hold a potential cure for T1D [36]. Yet, the current approach subjects patients to high-risk conditions, using chemotherapies that cause temporary immunodeficiency and potentially life-threatening infections. Efforts are undertaken to try and achieve a subtler reprogramming of the unwanted immune reaction in a tissue-specific manner, thereby reducing risk of the procedure while maintaining its efficacy. As T1D is a heterogeneous disease, a (semi-) personalized approach is warranted for the selection of appropriate candidates and appropriate immune endpoints for these novel therapies. Detailed knowledge on the genetic background of affected individuals and their exposure to environmental risk modulators can be used to 'match' the patient to the correct treatment and reduce the unnecessary exposure of patients to therapies that are unlikely to provide them any benefit. Determining biomarkers that distinguish T1D patients from healthy individuals, genetic factors that can mediate environmental triggers to altered immune status and genetic variants that are causal for the association of T1D loci

with disease, as described in chapter 3, chapter 4 and chapter 5 of this thesis, will benefit patient selection and subsequent personalized treatment. Therefore, it is imperative that it remains a focus of attention alongside the development of novel therapeutic strategies. Currently several approaches using recombinant antibodies to immune-receptors, adoptive regulatory T cell therapy or vitamin D modified dendritic cells are in progress, and might prove efficacious and feasible options for the treatment of T1D. As with anything the proof of the pudding is in the eating, and results of these trials are eagerly awaited.

Other strategies for combatting T1D aim to restore the insulin deficit directly, and thereby glycemic control, in T1D, rather than correcting the autoimmune response. The development of automated, closed-loop systems that mimic the pancreas' ability to sense glucose and release insulin according to need promises improved glycaemic control and increasing quality-of-life for T1D patients. Although this provides great promise in terms of improved quality of life and reduces the chance of acute complications of insulin replacement therapy, the artificial pancreas is not expected to fully reach the accuracy of endogenous β cells and will probably not completely prevent the long-term complications of T1D. Advances in (islet) transplantation have made replacement of lost β cells a realistic option for T1D patients, although as with all transplantations this approach suffers greatly from the shortage of donor organs and side-effects associated with immunosuppressive drugs that are, ironically, toxic to β-cells. Furthermore, as autoimmunity has a long-lived memory, the disease often recurs, leaving patients again dependent on exogenous insulin administration. The development of islet-protecting encapsulations, that allows for the free transfer of insulin while preventing the destruction of transplanted islets, promises better graft survival and transplant success. Concurrently, new sources of β cells are being investigated and with recent breakthroughs in differentiation of embryonic stem cells to (neo) beta-cells and establishment of immortalized β cell lines renewable sources of β cells appear within reach.

With all of these approaches issues regarding safety, feasibility, efficacy and cost-effectiveness need to be addressed and the continuing efforts of all those involved in overcoming T1D will be needed for the years to come.

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NEDERLANDSE SAMENVATTING

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DANKWOORD

NEDERLANDSE SAMENVATTING

Type 1 diabetes mellitus (T1D) is een auto-immuunziekte waarbij β-cellen in de alvleesklier worden vernietigd door een nog onvolledig begrepen auto-immuunreactie. β- cellen zijn sterk gedifferentieerde neuro-endocriene cellen en de enige bron van het glucose-regulerende hormoon insuline. Het verlies van β-cellen leidt in patiënten met T1D tot een tekort aan absoluut insuline en daardoor aan inadequate glucoseregulatie. Zonder insuline zijn vet-, lever- en skeletspiercellen niet in staat glucose uit de bloedbaan op te nemen, wat zowel leidt tot een tekort aan de belangrijkste energiebron voor de perifere weefsels als tot een verhoogde bloedsuikerspiegel (hyperglycemie). Onder normale fysiologische omstandigheden wordt glucose door de nieren volledig uit de pre-urine geresorbeerd en komt het niet in de urine terecht. Bij ernstige hyperglycemie wordt het resorberend vermogen van de nieren voor glucose overtroffen en is glucose aantoonbaar in de urine (glycosurie). Dit fenomeen is verantwoordelijk voor de naam van T1D; diabetes mellitus betekent *zoete doorstroom* in het Grieks. Door het osmotische effect van glucose in de nierbuisjes veroorzaakt glycosurie een gelijktijdig verlies van grote hoeveelheden water en elektrolyten, wat het pathognomonische symptoom van veel urineren (polyurie) verklaart. Polyurie veroorzaakt op zijn beurt dehydratie en daarmee overdreven dorst wat het tweede pathognomische kenmerk van diabetes mellitus verklaart; polydipsia (veel drinken). In de diabetische toestand is glucose niet meer beschikbaar als energiebron voor perifere weefsels en stapt het lichaam over naar alternatieve mechanismen van energieproductie om vitale cellulaire processen te handhaven, zoals de oxidatie van vetzuren. Als bijproduct van de oxidatie van vetzuren worden zure ketonenlichamen. Het verlies van circulerend volume en elektrolyten die het gevolg zijn van de glycosurie, gecombineerd met de verhoogde productie van zure ketonenlichamen, kan leiden tot een levensbedreigende verstoring van het zuur-base evenwicht in het lichaam, een medische noodgeval dat wordt aangeduid met de *diabetische ketoacidose*. Als de metabole acidose niet snel wordt gecorrigeerd met intraveneuze toediening van vloeistoffen en exogeen insuline, leidt de aandoening tot een comateuze toestand van de patiënt en uiteindelijk de dood.

In een van de oudste medische geschriften, de Ebers Papyrus, wordt al een ziektebeeld met de typische kenmerken van T1D beschreven door de geneesheren uit het oude Egypte. Ondanks dat de ziekten al duizenden jaren bekend is, betekende het stellen van de diagnose een onherroepelijk doodvonnis voor de patiënt totdat Banting, Best en collegae in 1921 als eersten insuline wisten te isoleren uit de alvleesklier van een hond. Deze doorbraak baande de weg voor de eerste werkelijke behandeling van T1D, de insuline-vervangende therapie, waarmee een dodelijke ziekte veranderde in een chronische aandoening. Ondanks de vooruitgang van de kwaliteit van de behandeling, met name door de ontwikkeling van nieuwe insuline-analogen, verbetering van de toedieningsmethoden en de algehele verbetering van de gezondheidszorg, blijft insuline-vervangende therapie een symptomatische behandeling en geen

genezing. Patiënten met T1D blijven levenslang afhankelijk van exogene insulinetoediening en lopen daarmee continue gevaar op de acute complicaties van deze therapie. Een teveel aan toegediende insuline leidt tot een te laag bloedsuikerniveau (hypoglycaemie) en indien ernstig genoeg een hypoglycemisch coma. Een tekort aan toegediende insuline leidt tot een diabetische ketoacidose. Beiden complicaties kennen een mogelijk dodelijke afloop. Naast de acute complicaties van hun behandeling lopen T1D patiënten het risico om op lange termijn complicaties als oog- hart- nier- en zenuwaandoeningen te ontwikkelen. Dit komt doordat insuline-vervangende therapie qua glucoseregulatie helaas de precisie mist van β-cellen. Door de behandeling zelf, die bestaat uit meerdere keren per dag glucose meten middels en het toediening van insuline, en de complicaties van hun behandeling, ervaren T1D patiënten een verminderde kwaliteit van leven evenals een verminderde levensverwachting. Opheldering van het ziekteproces van T1D is noodzakelijk voor de ontwikkeling van een definitieve genezing en daarmee een gewild doel in de moderne geneeskunde. Momenteel wordt het ziekteproces van T1D nog onvolledig begrepen, maar bekend is dat zowel omgevingsfactoren als genetische eigenschappen bijdragen aan het risico om de ziekte te ontwikkelen. Hierbij is er een grote heterogeniteit in ziekteprogressie, waarmee het waarschijnlijk is dat er waarschijnlijk verschillende optimale therapieën bestaan voor verschillende patiënten. Het ophelderen van de genetische mechanismen die bij de ziekteontwikkeling betrokken zijn zal bijdragen aan gepersonaliseerde geneeskunde en is belangrijk voor de ontwikkeling van nieuwe therapieën en een mogelijke curatieve behandeling. Het doel van dit proefschrift is hieraan bij te dragen door nieuwe inzichten te geven in zowel transcriptionele als post-transcriptionele genregulatie in het ziekteproces van T1D.

Hoofdstuk 2

In hoofdstuk 2 van dit proefschrift wordt de rol van differentiële splicing van *G6PC2* tussen alvleesklier en de thymus bij de ontwikkeling van auto-immuniteit beschreven. Het gen *G6PC2* codeert voor het eiwit islet-specific glucose-6-posphatase catalytic subunit-related protein (IGRP), een bekend doelwit van autoreactieve $CD8^+$ T cellen in patiënten met T1D. In een eerdere wetenschappelijke publicatie werd gesuggereerd dat bepaalde isovormen van *G6PC2*, met name degene die exon 3 en 4 van *G6PC2* bevatten, niet in de thymus tot expressie komen en dat dit in bijdraagt aan de autoimmuun-reactie tegen IGRP. Om te onderzoeken wat het daadwerkelijk effect van differentiële splicing op auto-immuniteit zou zijn hebben we eerst isovorm-specifieke primers voor G*6PC2* ontwikkeld om de expressie van *G6PC2* in de thymus en alvleesklier te kunnen kwantificeren. Hiermee toonden we aan dat, in tegenstelling tot eerdere aannames, alle conventionele *G6PC2* isovormen in de thymus te vinden zijn, al ware het in aanzienlijk lagere hoeveelheden dan in de alvleesklier. Vervolgens hebben we de autoreactiviteit tegen de verschillende *G6PC2* isovormen onderzocht door het niveau van IGRP-specifieke autoantilichamen en de frequentie van IGRP-specifieke, HLA-A2 gerestricteerde $CD8^+$ T-cellen in het perifere

bloed van de patiënten en gezonde personen te bepalen. Ondanks dat T-cellen die reactief zijn tegen IGRP-afgeleide peptiden gemakkelijk gedetecteerd konden worden in het perifere bloed van zowel gezonde individuen als T1D patiënten, wat op zichzelf suggereert dat negatieve selectie van reactieve T-lymfocyten van IGRP onvolledig is, werd er geen significant verschil gevonden tussen T1D patiënten en gezonde individuen. Deze bevinding impliceert dat de defecte negatieve selectie van IGRP-reactieve T-cellen opzichzelfstaand onvoldoende is voor de ontwikkeling van T1D. Concluderend corrigeerden we een eerdere aanname dat er sprake is van kwalitatief verschil in splicing van *G6PC2* tussen alvleesklier en thymus. Verder toonden we aan dat de negatieve selectie van IGRP specifieke T cellen inherent incompleet is, maar geen directe gevolgen heeft voor de ontwikkeling van auto-immuniteit in het kader van T1D.

Hoofdstuk 3

Zoals reeds beschreven werd in hoofdstuk 2 worden auto-reactieve T-cellen frequent aangetroffen in het perifere bloed van gezonde personen. Een mogelijk verschil tussen niet-aangedane individuen en patiënten zou de capaciteit van hun regulerende T-cellen (T-reg cellen) kunnen zijn om auto-immuunresponsen te dempen of überhaupt te voorkomen dat ze plaatsvinden. Zowel verminderde activiteit van T-reg cellen, als een verhoogde weerstand auto-reactieve T-cellen voor regulatie zijn beschreven voor verschillende auto-immuunziekten, waaronder T1D. In hoofdstuk 3 van dit proefschrift vergeleken we twee auto-reactieve T-cel klonen geïsoleerd uit een T1D patiënt met een auto-reactieve T-cel kloon geïsoleerd uit een gezond individu. Daarbij constateerden we dat auto-reactieve T-cellen van het gezonde individu maar een beperkt aantal keer konden worden gestimuleerd totdat ze stopten met vermenigvuldigen, een kenmerk dat niet waargenomen werd in auto-reactieve T-cellen van de T1D patiënt. Transcriptoom analyse van de verschillende T-cel klonen onthulde in de T-cellen van de T1D patiënt gereduceerde expressie van TRAIL, TRAIL-R2, FAS en FASLG, genen betrokken bij het proces van apoptose (gereguleerde celdood). De gereduceerde expressie van deze genen ging gepaard met verhoogde expressie van een drietal microRNA's, moleculen met een belangrijke rol in de post-transcriptionele regulatie van genexpressie. Computermodellen voorspelden dat de microRNA's miR-98, miR-23b en miR-590-5p betrokken waren bij de regulatie van TRAIL, TRAIL-R2, FAS en FASLG. Eerst leverden we het bewijs dat de microRNA's miR-98, miR-23b en miR-590-5p daadwerkelijk de genen TRAIL, TRAIL-R2, FAS en FASLG reguleerden met behulp van dual-luciferase reporter assays. Vervolgens transfecteerden we de verschillende microRNA's in primaire T-cellen waarbij een significant verminderde expressie van FAS- en TRAIL-mRNA-expressie werd waargenomen. Hiermee werd de functionele relevantie van deze microRNA's bij de weerstand van auto-reactieve T-cellen tegen apoptose onderstreept. Omdat de expressie van microRNA's beïnvloed kan worden door milieufactoren, zou verdere analyse van het microRNA transcriptoom van auto-reactieve cellen mogelijk nieuwe therapeutische opties kunnen verschaffen en

inzicht kunnen verschaffen in omgevingsfactoren die betrokken zijn bij de ontwikkeling van T1D. Samenvattend toonden we aan dat de verschillen in proliferatieve capaciteit tussen auto-reactieve T-cellen van gezonde individuen en patiënten samenhangt met verschillen in expressie van pro-apoptotische genen en de post-transcriptionele factoren die ze reguleren.

Hoofdstuk 4

In hoofdstuk 4 van dit proefschrift onderzochten we de mogelijke interactie tussen zeldzame genetische varianten en functionaliteit van microRNA's. Genoom brede associatiestudies (GWA's) hebben onze kennis over de relatie van genomische gebieden en het voorkomen van (auto-immuun)ziekten sterk vergroot. Echter, zelfs als men alle geverifieerde ziektegeassocieerde genomische regio's van T1D in aanmerking neem, is het onmogelijk de erfelijkheid van T1D volledig te verklaren. GWA's zijn ontworpen voor de detectie van frequent voorkomende genvarianten, zo geheten single nucleotide polymorphisms (SNP's). Daarom kunnen zeldzame SNP's en structurele DNA variaties als inserties, deleties en duplicaties niet geanalyseerd worden met de huidige generatie GWA's. Er wordt daarpm verondersteld dat de 'ontbrekende erfelijkheid' van complexe ziekten als T1D kan worden verklaard door zeldzame en structurele genvariaties. In een proof-of-concept-studie hebben we zeldzame polymorfismen in T1D-risicogenen *in silico* onderzocht op hun capaciteit om post-transcriptionele genregulatie te beïnvloeden door de binding van microRNA's te veranderen. Door middel van mutagenese testten we deze voorspellingen en toonden *in vitro* aan dat zeldzame SNP's, welke we miRSNP's noemen, het vermogen hebben microRNA-gemedieerde genregulatie te beïnvloeden door ofwel een bindingplaats voor microRNA te creëren, dan wel deze te verstoren. Hiermee geven we bewijs voor een nieuw mechanisme waarmee zeldzame genvariaties het risico op het ontwikkelen van T1D kunnen beïnvloeden. Omdat de expressie van microRNA's beïnvloed kan worden door omgevingsfactoren, omschrijft deze studie tevens een manier waarop omgevingsfactoren selectief voor individuen met bepaalde DNA eigenschappen het risico op T1D kunnen vergroten. Echter zal er eerst meer bekend moeten worden over de exacte manier waarop de expressie van microRNA's zelf gereguleerd wordt voordat er duidelijke verbanden uit getrokken kunnen worden tussen omgevingsfactoren, zeldzame DNA variaties en de ontwikkeling van T1D.

Hoofdstuk 5

Cytotoxische T-lymfocytenantigen-4 (CTLA-4) is een oppervlaktemolecuul aanwezig op geactiveerde T-cellen dat de T-celreceptorsignalering remt. Er wordt gedacht dat variaties binnen het *CTLA4*-gen het risico op de ontwikkeling van auto-reactieve T-cellen, en daarmee het risico op auto-immuunziekten als T1D, vergroot. Het *CTLA4* locus ligt op een genomisch gebied dat als een van de eerste werd geassocieerd met gevoeligheid voor de ontwikkeling van T1D. Toch bleef de genetische variant die verantwoordelijk is voor

deze associatie, evenals de moleculaire mechanismen die door deze de variatie beïnvloed werden, onbekend. Recente studies hebben aangetoond dat de regio gekenmerkt door de SNP CT60 (rs3087243) fungeert als risicofactor. Het CT60 polymorfisme bevindt zich echter niet in het *CTLA4*-gen, maar ligt *downstream* van het gen in een niet-coderend gebied. De manier waarop deze variatie de functie van het *CTLA4*-gen zou beïnvloeden is dan ook niet bekend. In hoofdstuk 5 van dit proefschrift tonen we aan dat een structurele genvariant, namelijk de $(AT)_n$ microsatelliet die zich in de 3'-untranslated region (UTR) van *CTLA4* bevindt, oorzakelijk is voor de genetische associatie van *CTLA4* met T1D gevoeligheid. Allereerst onderzochten we de associatie van de *CTLA4* $(AT)_n$ microsatelliet met de T1D risicomarker CT60. Deze analyse toonde aan dat CT60 status en $(AT)_n$ lengte zeer nauw samenhingen. *CTLA4* allelen met langere $(AT)_n$ elementen kwamen alleen gekoppeld met het risico haplotype CT60G voor. Omgekeerd werd het beschermende CT60A haplotype vaker gezien in samenhang met wildtype, korte $(AT)_n$ elementen. Derhalve correleerden lange $(AT)_n$ elementen direct met een verhoogd risico op β-cel auto-immuniteit. In auto-reactieve T-cellijnen associeerden langere microsatelliet lengten met een significant verminderde *CTLA4* genexpressie. Tevens leidde de transfectie van een lange $(AT)_n$ microsatelliet *in vitro* tot verlaagde reporter mRNA-expressie van een *reportergen*. Het directe effect van de lengte van de $(AT)_n$ microsatelliet op genexpressie, in combinatie met de uitzonderlijk sterke correlatie met CT60 status impliceert een causale rol voor de $(AT)_n$ microsatelliet in de genetische associatie van het *CTLA4* gebied met T1D gevoeligheid. Tevens geeft deze een verklaring voor het mechanisme waarmee de variatie de gevoeligheid voor T1D verhoogd, namelijk door de expressie van het immuunregulerende gen *CTLA4* te verminderen.

TOEKOMSTPERSPECTIEVEN

Onderzoek

De afgelopen decennia hebben (bio)medische wetenschappers een overvloed aan informatie over de genetica van complexe ziekten zoals T1D vergaard. Inzicht krijgen in hoe genvarianten ziektegevoeligheid beïnvloeden en de interactie tussen milieu, genetica en immunologie bepalen, zijn de volgende hindernissen om te nemen. Het bereiken van deze doelen kan bijdragen aan het voorspellen van ziekteprogressie, ontwerpen van nieuwe behandelmethoden en de ontwikkeling van "gepersonaliseerde geneeskunde". Dit werk heeft slechts een klein deel van de vele aspecten van de functionele genetica van T1D behandeld. Met recente technologische vooruitgangen is *whole genome sequencing*, een techniek die het volledige genoom van een enkel individu compleet in kaart brengt, binnen handbereik gekomen voor onderzoekers. In de nabije toekomst zal het hiermee mogelijk zijn álle genetische variatie van een individu tegelijkertijd te bepalen, en daarmee de beperkingen van GWA's wegnemen. Tegelijkertijd zal deze aanpak een ongeëvenaarde hoeveelheid complexe data genereren. Waarschijnlijk zullen gegevensanalyse en -verwerking samen met de statistische analyse hiervan een volgend knelpunt zijn.

Nieuwe strategieën voor gegevensbeheer en analyse zijn dus noodzakelijk. Uit de celbiologisch perspectief zal het onderzoek naar T1D hoogstwaarschijnlijk profiteren van zogeheten *single-cell* technieken die momenteel (door)ontwikkelt worden. Deze technieken maken isolatie en analyse van auto-reactieve cellen direct uit het bloed van patiënten mogelijk en verminderen daarmee de kans op artefacten die komen kijken bij de, nu nog noodzakelijke, celkweken. Bovendien biedt het direct isoleren en analyseren van auto-reactieve cellen de mogelijkheid voor "high-throughput" analyses. Gecombineerd met verbeteringen in data-analyse kan deze aanpak van onschatbare waarde zijn bij het identificeren van de moleculaire mechanismen die betrokken zijn bij, of mogelijk zelfs diegene die voorafgaan aan, β-celvernietiging.

Behandeling

Momenteel zijn er veelbelovende vooruitzichten voor de behandeling van T1D, waaronder de kunstmatige alvleesklier, van stamcellen afgeleide (neo) β-cellen en immuuntherapieën. Recente myeloablatieve therapieën gevolgd door latere autologe stamceltransplantatie resulteerden in gereduceerde insulinebehoefte en in sommige gevallen zelfs langdurige insulineonafhankelijkheid. Deze aanpak stelt patiënten echter bloot aan hoge risico's, aangezien chemotherapie een noodzakelijk van deze behandeling is. Momenteel wordt er in verschillende onderzoekgroepen gewerkt aan een subtielere manier de ongewenste immuunreactie op een weefselspecifieke manier te "herprogrammeren". Aangezien T1D een heterogene ziekte is, is een (semi-) gepersonaliseerde benadering in de toekomst gerechtvaardigd bij de keuze voor de geschikte (immuun)therapie. Gedetailleerde kennis over de genetische achtergrond en de persoonlijke blootstelling van omgevingsfactoren kan worden gebruikt om per patiënt de juiste behandeling te kiezen en kan onnodige blootstelling aan ineffectieve therapieën voorkomen. Onderzoek zoals beschreven in dit proefschrift heeft de potentie om gepersonaliseerde behandeling ten goede komen. Derhalve is het van essentieel belang dat naast de ontwikkeling van nieuwe therapeutische strategieën in de toekomst, ook aandacht wordt besteed aan de (functionele) genetica van T1D.

Naast onderzoeken die de genezing van T1D nastreven zijn er verschillende initiatieven om de huidige behandeling van T1D te verbeteren. De ontwikkeling van geautomatiseerde systemen met een "closed-loop", die het vermogen van de alvleesklier om zelfstandig glucose te detecteren en de benodigde insuline vrij te geven nabootsen, belooft verbeterde glycemische controle en verhoogde levenskwaliteit voor T1D patiënten. Hoewel de kunstmatige alvleesklier de nauwkeurigheid van endogene β-cellen waarschijnlijk niet volledig zal bereiken en daarmee dus ook de lange-termijncomplicaties niet geheel zal kunnen voorkomen heeft deze ontwikkeling wel de potentie om de levenskwaliteit van T1D te verbeteren en de risico's op acute complicaties van insulinetoediening te reduceren. Transplantatie van β-cel bevattende eilandjes van Langerhans lijkt door recente vooruitgangen een realistische behandeloptie voor T1D patiënten, maar zoals

bij alle transplantaties lijdt deze aanpak sterk onder het tekort aan donororganen en bijwerkingen van de afweeronderdrukkende geneesmiddelen die noodzakelijk zijn na transplantatie. De ontwikkeling van beschermende capsules die de getransplanteerde eilandjes van Langerhans beschermen, belooft betere transplantaatoverleving en daarmee succes. Tegelijkertijd worden er momenteel nieuwe bronnen van β-cellen onderzocht en met recente doorbraken in de differentiatie van embryonale stamcellen naar (neo) β -cellen en de ontwikkeling van kweekbare β-cellijnen komen hernieuwbare bronnen van β-cellen binnen bereik.

Bij al deze benaderingen is de samenwerking tussen (bio)medici, industrie, overheid en vrijwilligers essentieel om problemen op het gebied van veiligheid, haalbaarheid, efficiëntie en kosteneffectiviteit aan te pakken en zal de continue inspanning van alle betrokkenen nodig zijn om de genezing van T1D te bewerkstellingen.

CURRICULUM VITAE

Vincent Martijn de Jong, roepnaam Martijn, werd op 8 april 1986 geboren te Rotterdam. In 2004 voltooide hij zijn VWO opleiding aan de Christelijke Scholen Gemeenschap de Goudse Waarden in de profielen Natuur & Gezondheid en Natuur & Techniek en begon hij met zijn studie Geneeskunde aan de Universiteit van Leiden. Naast zijn studie was hij werkzaam op de Thoraxchirurgie Intensive Care in het LUMC en bij het Center for Human Drug Research te Leiden. Naast zijn studie Geneeskunde voltooide hij in 2008 de pre-Master Biomedische Wetenschappen, om in 2009 aan de Master Biomedische Wetenschappen te beginnen. In 2011 ontving hij de MD/PhD promotiebeurs voor excellente studenten en startte hij naar aanleiding van zijn wetenschapsstage een promotietraject onder prof.dr. B.O. Roep aan de afdeling Immunohematologie en Bloedtransfusie. Na afronding van het praktische deel van zijn promotieonderzoek in 2013, keerde hij terug als student om zijn coschappen en Master af te ronden. In 2014 behaalde hij *cum laude* zijn artsexamen en Master in de Biomedische Wetenschappen. Na zijn studie werkte Martijn als arts-assistent niet in opleiding tot specialist (ANIOS) op de Interne Geneeskunde, Longziekten en Cardiologie in het Rijnland Ziekenhuis, tegenwoordig Alrijne Ziekenhuis, te Leiderdorp. Vanaf 1 januari 2017 is hij werkzaam als op de afdeling Cardiologie binnen het HagaZiekenhuis te Den Haag, waar hij per 1 januari 2018 begonnen is aan zijn opleiding tot cardioloog onder opleider dr. J.W.J. Vriend.

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