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# Insulin: a critical autoantigen and potential therapeutic agent in Type 1 diabetes

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**Insulin is a polypeptide hormone secreted by pancreatic  $\beta$ -cells and is critical for glucose homeostasis. Abnormalities in insulin secretion result in various forms of diabetes. Type 1A diabetes is an autoimmune form in which insulin has been identified as a critical autoantigen. Recent studies have identified genetic determinants of insulin-specific autoimmune responses and insulin epitopes targeted by autoreactive T lymphocytes. The study of insulin as an autoantigen has also led to discoveries about basic mechanisms of immunological tolerance and autoimmunity. Experimental and clinical evidence suggests that insulin and insulin-derived peptides may delay and perhaps prevent the development of diabetes. Further clinical trials may identify effective treatment modalities for inhibiting diabetogenic autoimmunity and preventing disease development.**

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## Insulin: a key hormone

Insulin is a polypeptide hormone secreted by pancreatic  $\beta$ -cells. It is produced as a pre-prohormone, pre-proinsulin, which is processed to proinsulin and then mature insulin. Human proinsulin consists of three components: the B-chain (30 aa residues), the connecting or C-peptide (35 aa), and the A-chain (21 aa). Mature insulin is obtained following the cleavage of the C-peptide by specific enzymes, while two disulphide bridges link the A and B chains. Insulin is synthesized and stored in the secretory granules of pancreatic  $\beta$ -cells; it is secreted quickly in the circulation in response to increasing blood glucose levels. The binding of the hormone to the insulin receptor, expressed by most nucleated cells and especially in cells of the liver, muscle and adipose tissue, results in a series of molecular events and biochemical reactions that promote glucose metabolism and maintain glucose homeostasis. Lack of insulin production or abnormalities affecting insulin secretion are key to the development of all forms of diabetes. Type 1A diabetes is an autoimmune form of diabetes. This review outlines the role of insulin as an autoantigen, as well as key molecular aspects of insulin autoimmunity

and its regulation that are relevant to the understanding of autoimmunity in general, and the potential of therapeutic approaches based on insulin and insulin-derived peptides to antagonize autoimmunity.

## Type 1 diabetes: an autoimmune disease Natural history

Insulin-dependent diabetes mellitus (IDDM), or Type 1 diabetes, is classified into two main subtypes: Type 1B diabetes has idiopathic etiology, while Type 1A diabetes is an autoimmune disease. The latter will be discussed in this review. Type 1A diabetes develops mostly in children and young adults. It may also develop in older individuals and approximately 10% of patients with Type 2 diabetes have evidence of anti-islet autoimmune responses [1]. Diabetes symptoms result from the T-cell-mediated, selective destruction and almost complete loss of pancreatic  $\beta$ -cells. Autoimmune responses and  $\beta$ -cell destruction usually proceed undetected for months or years and precede the onset of clinical symptoms, which appear when insulin secretion is dramatically impaired, reflecting severe  $\beta$ -cell loss. However, significant numbers of  $\beta$ -cells are estimated to still be present at disease

onset [2]. These cells may be functionally impaired and may recover during the following months, so that some patients experience a brief period of insulin independence, the honeymoon period. This is followed by the return to permanent insulin dependence, although evidence for some degree of regeneration was reported recently in patients with long-standing diabetes [3].

### Disease pathogenesis

Over the last few decades there has been much progress in understanding the genetic and immunological roots of the disease. Although most patients lack a family history of disease, Type 1A diabetes develops predominantly in individuals with a genetic predisposition and has significant familial clustering. Strong associations have been established with genes located on the short arm of chromosome 6, in particular those coding for both class I and class II human leukocyte antigen (HLA). Specific allelic variants at the class II *HLA-DR* (DR3, DR4) and *HLA-DQ* (DQ2, DQ8) loci have been identified as main susceptibility determinants [4]. A number of other genes and less characterized loci have been implicated in disease susceptibility [5], including the insulin gene (*INS*) on chromosome 11p15 [6].

Humoral autoimmune responses are clearly associated with disease development. The discovery by Bottazzo and colleagues – that serum from Type 1A diabetes patients reacts with pancreatic islets – led to the identification of islet cell antibodies (ICA) as an important diagnostic and risk marker for diabetes development [7]. Studies showed that ICA preceded diabetes development and were strong disease predictors, opening the way for prevention studies in first-degree relatives with increased risk, defined by the presence of ICA [8]. Further progress led to the identification of autoantibodies to specific molecules in the patients' sera. Similar to ICA, these autoantibodies are most commonly observed during the prodromic period and around the time of disease onset, while titers tend to decline in most, but not all patients, after diagnosis [9]. Assays for measuring autoantibodies are well established and are used routinely to assess diabetes risk in clinical trials [10], as the presence of multiple autoantibodies correlates with higher disease risk. Besides marking diabetes risk, there is increasing evidence that autoantibodies and B lymphocytes participate in the molecular mechanisms leading to  $\beta$ -cell destruction, both from studies *in vitro* [11] and from animal models [12].

However,  $\beta$ -cell destruction is mainly T-cell mediated, with both CD4 and CD8 T cells participating in the disease process and complementing their pro-inflammatory actions [13]. T cells are the predominant cell type in the lymphocytic infiltrate that is observed in the pancreatic islets during the prodromic period leading to disease development. This inflammatory process, known as insulinitis, is followed by severe damage to the islet structure and over time results in selective  $\beta$ -cell loss [14]. Cytokines and other inflammatory mediators play critical roles in recruiting and modulating the function of the infiltrating T cells, and some cytokines can also directly damage  $\beta$ -cells and impair many of their vital functions [15].

A variety of molecules have been identified as autoantigens in Type 1 diabetes [16]. The autoimmunity against pancreatic  $\beta$ -cells is probably heterogeneous. Not all patients have autoimmune responses against the same autoantigens or epitopes. Autoimmune responses, especially T-cell responses, may not necessarily be present at the same time but rather they may be triggered at different time points. Thus, T-cell responses may come in several temporal waves, so that their relationship to disease progression may be missed unless frequent monitoring is utilized. Clearly, the ability to reliably measure and prospectively follow T-cell responses, and to define their relationship with disease development, has been limited. Serum autoantibodies are easily and more reliably measured, but it is unclear how their levels correlate with T-cell responses. Specific data on whether peripheral blood T-cell reactivity correlates well with disease progression in the pancreas, and how and whether changes in the frequency and phenotype of autoreactive T cells in peripheral blood reflect changes in disease activity, are lacking, with data too limited, even in mouse models [17].

This lack of knowledge is owing in large part to the lack of robust T-cell assays and to the fact that most studies have analyzed peripheral blood samples. It is indeed very difficult to obtain and study human pancreata with ongoing  $\beta$ -cell autoimmunity, and thus studies of the actual pancreatic lesions have been limited by poor access to the pancreas of prediabetic subjects or new onset patients [18]. Biopsy of the pancreas in prediabetic subjects and patients has been performed in Japan [19], providing highly valuable information but also a limited amount of tissue, which may be insufficient for in-depth investigations and the development of robust criteria. Much greater access to pancreas and pancreatic lymph nodes is possible through screening pancreas organ donors for autoantibodies, as recently reported by Gianani and colleagues [20]. Additional, complementary sources of pancreata and more substantial biopsy material may be obtained from pancreas transplant recipients with recurrent or persistent autoimmunity [21]. Coordinated efforts to address these problems appear critical at this juncture and are likely to result in useful information, since sophisticated techniques to study the pancreatic lesion and to characterize T-cell specificities and phenotypes are becoming increasingly available.

### Insulin: a critical autoantigen

#### Insulin autoantibodies

It has been almost 25 years since insulin was identified as an autoantigen following the discovery that serum from patients with Type 1A diabetes, collected at disease onset and before insulin treatment, contained insulin autoantibodies [22]. The development and refinement of immunoassays for measuring insulin autoantibodies (IAA) allowed for more extensive studies in patients and first-degree relatives: IAA were found to precede disease onset and to correlate with disease risk in first-degree relatives [23]. IAA are particularly frequent in younger children and IAA levels inversely correlate with age [24]. IAA levels are also associated with HLA-DR4, one of the main genetic determinants of disease risk [25], and with certain HLA-DQA1

alleles [26]. Newborn screening programs allow identification of individuals who are genetically at risk, who are then followed from birth for autoantibody development. Data from such programs (Diabetes AutoImmunity Study in the Young [DAISY], BABYDIAB and Melbourne Prediabetes Study) suggest that IAA very often, but not always, precede the development of other autoantibodies [27–31].

In the BABYDIAB study, which follows the offspring of parents with Type 1A diabetes from birth, high-affinity IAA were associated with HLA-DR4, young age of IAA appearance and subsequent progression to multiple islet autoantibodies or overt disease. Importantly, IAA affinity in children with multiple autoantibodies was on average 100-fold higher than in children who only expressed IAA positive or became autoantibody negative on follow-up. Unlike low-affinity IAA, high-affinity IAA required conservation of human insulin A-chain residues 8–13 and were reactive to proinsulin. These data suggest that early sensitization to proinsulin and perhaps the 8–13 epitope on the A chain is associated with spreading of islet autoimmunity and progression to diabetes [32]. Studies in the nonobese diabetic (NOD) mouse, a model of spontaneous autoimmune diabetes closely resembling the human disease [33], also demonstrate that IAA are associated with disease development. Although the NOD is an inbred strain, there is heterogeneity in IAA frequency, levels and time of appearance. However, the presence of IAA at 8 weeks of age strongly correlates with disease development [30].

### Insulin T-cell epitopes

Studies in the NOD mouse demonstrate that T-cell responses to insulin usually precede autoimmunity to other molecules [34]. Similarly, T-cell responses to the B24–C33 residues of proinsulin also appear early compared with responses to other antigens [35]. Autoimmune responses to a B-chain epitope encompassing the B9–23 residues (B9–23 for CD4 T cells, B15–23 for CD8 T cells) have been associated with diabetes in NOD mice and represent a major component of the T-cell infiltrate [36,37]. In addition, immunization with the B9–23 peptide induces IAA and insulinitis in mice treated with a viral mimic agent (polyinosinic-polycytidylic acid). Furthermore, diabetes develops if, in addition, mice transgenically express co-stimulatory B7-1 molecules in the islets [38–40]. Thus, immunization with the insulin B9–23 peptide can stimulate diabetogenic autoimmune responses even in mice that are not genetically prone to disease, such as BALB/c and NOR mice.

In humans, there is similar evidence that T-cell responses to insulin and proinsulin are associated with Type 1A diabetes [13]. Responses to epitopes of proinsulin/insulin reported in patients and prediabetic subjects include those to proinsulin epitopes B24–C36 [41], C18–A1, B11–C24, and C28–A21 [42,43]. CD4 T-cell responses to the main epitope targeted in NOD mice (B9–23) have also been reported in patients and prediabetic subjects [44]. In addition, CD8 T-cell responses are reported in patients against several epitopes of the B-chain and the B-chain–C-peptide junction, including B24–C36 [45]. Many patients reacted against multiple

pro-insulin epitopes; responses to some CD8 epitopes were restricted to HLA-A1 and HLA-B8, but several epitopes bound to several HLA class I molecules [45]. Additional pro-insulin epitopes that might be targeted in patients with Type 1A diabetes were recently identified by reverse immunology [46]. HLA-DR4-restricted, CD4 T cells to an A-chain epitope, the A1–15 epitope, were reported following autopsy in the pancreatic lymph nodes of patients with long duration of diabetes [47], demonstrating insulin-reactive T cells in close physical proximity to the target organ. T cells plausibly reacting to the same epitope (A1–13) were also detected in an HLA-DR4-positive prediabetic child with IAA [48]. Of note, the sequence of this epitope partially corresponds to the A8–13 epitope targeted by high-affinity IAA in children prior to the development of diabetes [32]. Immunity to the A1–15 (or A1–13) T-cell epitope results from post-translational modification of this epitope [48]. T-cell recognition is dependent on a vicinal disulfide bond between adjacent cysteines at the A6 and A7 residues, normally not present in the insulin molecule. Because T cells generated using a modified A1–13 peptide also reacted to islet cell lysates, this modified epitope must be present *in vivo*, either produced in islets or by antigen-presenting cells (APCs). These findings further support the concept that insulin may act as a triggering autoantigen if post-translational modification results in an epitope that the immune system was not tolerized to. Whether or not similar modifications occur for other autoantigens has not been investigated.

### Insulin expression in relation to disease pathogenesis

The three most common diabetes autoantigens are all expressed in pancreatic  $\beta$ -cells. Besides insulin, the two best characterized autoantigens are glutamic acid decarboxylase (GAD) and a tyrosine phosphatase-like protein (IA-2 or ICA512) [49]. The 65-kDa GAD isoform is an enzyme that converts glutamate into  $\gamma$ -amino butyric acid. It is a cytosolic protein, which in  $\beta$ -cells is associated with synaptic-like secretory vesicles resembling neuronal synaptic vesicles, where GAD is also expressed. IA-2 is an intrinsic membrane protein of the  $\beta$ -cell secretory granules, which is expressed in virtually all neuroendocrine cells. IA-2 is involved in the insulin secretion machinery and regulates the numbers of secretory vesicles and basal insulin secretion [50]. Another autoantigen in the NOD mouse, the islet-specific glucose-6-phosphatase-related protein (IGRP), is also  $\beta$ -cell specific. Evidence suggesting that IGRP may be an autoantigen in human diabetes has recently been published [51], although further studies appear necessary to establish this firmly.

Insulin has been considered to be the only  $\beta$ -cell-specific antigen because its expression has long been thought to be exclusively restricted to pancreatic  $\beta$ -cells. Moreover, insulin is the autoantigen most abundantly produced by  $\beta$ -cells, being expressed at levels that are several thousand-fold that of GAD and IA-2. Such an increased abundance is likely to increase the probability that insulin may be post-translationally modified, which, as noted above, may result in the formation of new epitopes recognized by autoreactive T cells [48]. It is also known

that insulin can be misfolded under certain conditions, a process that is favored by apoptosis and endoplasmic reticulum stress, phenomena that in turn have been linked to diabetes development in experimental models [52–54]. However, other tissues/cells can actively transcribe *INS* and produce low levels of (pro)insulin, including the fetal central and peripheral nervous systems [55], the adrenal [56], the retina [57], the yolk sac [58] and the gut [59]. (Pro)insulin production in these extra pancreatic tissues does not contribute to the pool of the circulating hormone; rather, it is involved in autocrine and paracrine effects, neuromodulation and other molecular pathways unrelated to glucose metabolism. (Pro)insulin expression by specific cells found in thymus and peripheral lymphoid organs appears to be critical for diabetes development [60].

#### **Insulin expression in the thymus & peripheral lymphoid tissues**

(Pro)insulin has been one of the earliest and prototypic molecules with tissue-restricted expression reported to be expressed in the thymus. It is now known that a large number of self-molecules with tissue-restricted or mainly peripheral expression are expressed in the thymus through the transcription of their corresponding genes [60]. These molecules include pancreatic and thyroid hormones, neuroendocrine molecules and many other proteins. In several instances, these proteins are known target autoantigens in autoimmune disease, including proinsulin/insulin, GAD and IA-2.

Several mechanisms controlling gene expression can influence the quantity and quality of self-molecule expression in the thymus [60]. Quantitative and qualitative differences in the expression of self-molecules may, in turn, affect the thymic selection and other mechanisms that require self-antigen presentation for achieving and maintaining self-tolerance. Importantly, *INS* transcription and (pro)insulin expression are also observed in peripheral lymphoid tissues [61–63]. Besides thymic epithelial cells [64], bone marrow-derived APCs, mainly CD11c-positive dendritic cells (DCs), transcribe *INS* and have also been shown to express a proinsulin epitope (B24-C36) on the cell surface in the thymus, peripheral lymphoid tissues and blood [63]. The transcription-dependent expression of this proinsulin and other self-molecule epitopes by DCs may play a key role in tolerance as well as susceptibility to autoimmunity.

#### **Genetic regulation of insulin expression in lymphoid organs**

The levels of insulin mRNA expression in the human thymus correlate with allelic variation at the *IDDM2-INS* susceptibility locus [65,66]. This locus corresponds to a polymorphic, variable nucleotide tandem repeat (VNTR) sequence upstream of the *INS* sequences [67]. The VNTR mediate a transcriptional signal in the steady state. The transcriptional activity in the thymus is approximately 200–300% higher for *INS* transcripts encoded by *IDDM2* alleles associated with resistance to disease development; conversely, transcription is lower for transcripts encoded by diabetes-predisposing VNTR alleles [66,68]. Furthermore, there is evidence that parent-of-origin effects may affect the susceptibility associated with the *IDDM2* locus [6]. These

parent-of-origin effects may be mediated by parental imprinting, a mechanism that could suppress the expression of one of the two insulin genes in both thymus and peripheral lymphoid organs. There is indeed evidence that *INS* can be monoallelically expressed in the thymus, the spleen and in the yolk sac [6]. Remarkably, the nonexpressed allele in thymus and spleen was always the one in *cis* with the diabetes-protective *IDDM2* alleles. Thus, silencing transcription from these alleles and, in turn, dramatically reducing (pro)insulin production in the thymus may block the protective effect.

Overall, the risk of diabetes that is determined by allelic variation and parent-of-origin effects at the *IDDM2* locus appears dependent on the levels of *INS* transcription in the thymus. Differential transcriptional regulation may determine the amounts of (pro)insulin produced and in turn influence the efficiency of thymic selection processes involving (pro)insulin-specific autoreactive T cells. The increased transcription levels detected in thymus fit well with the dominant protective effect associated with certain *IDDM2* alleles, as higher insulin levels in the thymus may more efficiently induce negative selection of (pro)insulin-specific T lymphocytes (or improved selection of regulatory T cells). By contrast, homozygosity for diabetes-predisposing alleles determines lower insulin levels, which may be associated with a less efficient deletion of (pro)insulin-specific autoreactive T cells (or impaired selection of regulatory T cells). *INS* transcription in the human thymus also correlates with protein production [66,68], where proinsulin appears to be the main gene product [61]. Thymus cells expressing proinsulin are not likely to possess the refined machinery necessary to process proinsulin to mature insulin. Rather, it would seem likely that proinsulin might be processed into peptides for presentation to developing lymphocytes and tolerance induction to proinsulin/insulin.

#### **Manipulation of insulin expression in mouse models shows that insulin is a critical autoantigen & that thymic expression is central to diabetes development**

Studies in transgenic and knockout mice provide direct evidence that (pro)insulin thymic levels can dramatically affect the development of self-tolerance to (pro)insulin. Moreover, they provide evidence that, at least in the NOD mouse, differences between the (pro)insulin expressed in the thymus and that expressed in the pancreas can have a critical role in disease pathogenesis. These mouse models most often involve manipulations of the NOD mouse. In the NOD strain, insulinitis develops and is followed by diabetes in approximately 80% of the female and 35–40% of the male mice by 30 weeks of age. The target autoantigens of the autoimmune process in mice overlap with those in humans, as they include (pro)insulin, GAD and IA-2. As discussed, autoimmune responses to (pro)insulin are among the earliest to develop during the natural history of the disease. Unlike humans, mice have two nonallelic insulin genes, which are located on two different chromosomes, *Ins1* and *Ins2*. They are both expressed in pancreatic  $\beta$ -cells, although they encode for slightly different proteins, insulin I and insulin II. The most important models are discussed below and illustrated in FIGURE 1.

# Graded deficiency of thymic insulin expression

Although allelic variation is not observed in the highly-inbred NOD mice and mice do not have VNTR sequences, varying levels of (pro)insulin thymic expression have been replicated in mice lacking the expression of either one or two copies of the two insulin genes. While insulin production in pancreatic  $\beta$ -cells is largely unaffected because of compensatory mechanisms, thymic expression is dependent on the number of insulin gene copies present [69]. These studies established that of the two insulin genes, it is *Ins2* that is also expressed in the thymus. This model of thymic-graded insulin deficiency also showed a linear correlation between insulin gene copy numbers and (pro)insulin autoimmunity. Mice expressing low thymic (pro)insulin levels presented spontaneous T-cell peripheral reactivity to insulin and the C-peptide, whereas mice with normal levels showed no significant response [69]. This work provides functional evidence that thymic (pro)insulin levels play a key role in the selection of (pro)insulin-specific T cells and supports the concept that variation in

*INS* levels determined by allelic variation and imprinting phenomena at the *IDDM2-INS* locus is the mechanism by which this locus influences disease risk.

# Thymic overexpression of the insulin gene

The fact that NOD mice normally develop diabetes and autoimmune responses to proinsulin/insulin reflects the insufficient amounts of *Ins2* expression in the thymus, as NOD mice express approximately 50% less insulin in the thymus compared with BALB/c mice [70]. Another important line of support for the hypothesis that levels of (pro)insulin expression in the thymus are crucial for thymic selection and diabetes susceptibility came from studies that utilized NOD mice overexpressing the *Ins2* gene under the MHC class II promoter [71]. The increased levels of *Ins2* expression in the thymus of the transgenic mice compared with nontransgenic NOD mice were associated with complete prevention of insulinitis and diabetes. These results are consistent with the notion that increased levels of (pro)insulin expression in the thymus could result in a more efficient deletion

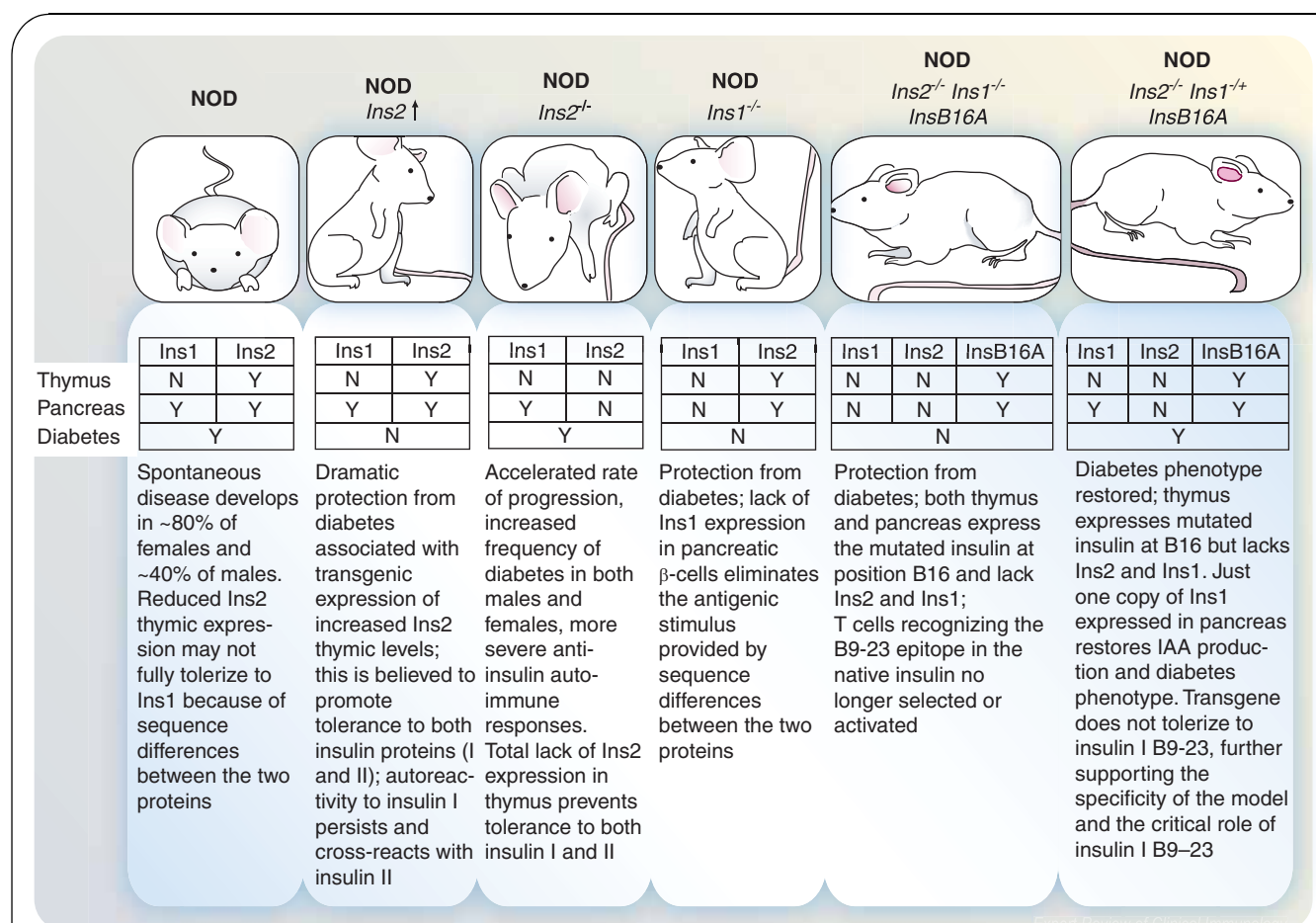


Figure 1. Schematic representation of the expression patterns of the mouse insulin genes in the thymus and pancreas. Mouse strains shown include the NOD mouse, which develops spontaneous autoimmune diabetes, and genetically manipulated models based on the NOD strain in which the expression of the insulin genes was altered. The figure illustrates the relationship between the pattern of insulin gene expression in the thymus and pancreas and the development of diabetes, and summarizes the main findings and interpretation of the models discussed in greater detail in the main text.

IAA: Insulin autoantibodies; NOD: Nonobese diabetic.

of (pro)insulin-specific T cells (FIGURE 1). The complete prevention of insulinitis in this model also suggests that insulin may play a critical role as an early autoantigen.

Insulin knockout models

Mice with a null *Ins2* mutation were bred onto the NOD background, so that most of the known susceptibility loci (including the major predisposing MHC locus, I-Ag7) were restored. NOD-*Ins2*<sup>-/-</sup> mice developed accelerated insulinitis and diabetes in two independent studies [72,73]; essentially all mice develop diabetes, regardless of sex, while it is known that male NOD mice are less prone to diabetes development. Moreover, NOD-*Ins2*<sup>-/-</sup> mice display increased IAA responses and splenocytes from these mice have increased capacity to transfer diabetes compared with splenocytes from wild-type NOD females [72]. By contrast, NOD mice lacking the expression of the *Ins1* gene are protected from insulinitis and diabetes [73]. Heterozygous mice also show decreased incidence of diabetes as well as delayed appearance of symptoms. NOD-*Ins1*<sup>-/-</sup> female mice express IAA similar to the wild-type NOD female mice, suggesting that IAA production may not be controlled by (pro)insulin expression in the thymus.

A plausible interpretation of the above findings is that the marked effects on diabetes susceptibility observed in *Ins2* and *Ins1* knockout NOD mice reflect differences between the two insulin proteins, which differ at positions 9 and 29 of the B-chain, and differences in the expression pattern between the thymus and pancreas (FIGURE 1, TABLE 1). These may be critical factors in the pathogenesis of diabetes in the NOD mouse. Since *Ins2* is normally the only insulin gene actively expressed in the thymus, NOD-*Ins2*<sup>-/-</sup> mice display accelerated diabetes because they do not have any (pro)insulin in the thymus to delete (pro)insulin-specific T cells. The protection from diabetes in NOD-*Ins1*<sup>-/-</sup> mice may be explained by sequence differences between (pro)insulin II, which is made in the thymus, and insulin I, which is made in the pancreas. In other words, the immune system may not be fully tolerant to insulin I because the thymus expresses only (pro)insulin II. Lack of (pro)insulin II completely abolishes tolerance to both insulin

proteins and accelerates diabetes, which is triggered by the expression of insulin I in the pancreas. The lack of insulin I expression only affects the pancreas, and diabetes may not develop because mice should be tolerant to (pro)insulin as this is expressed in the thymus.

A comparison of the sequences of the two proteins and the epitopes that have been identified as autoimmune targets in the NOD mouse reveals that, with the exception of a single epitope encompassing the A-chain, all epitopes overlap with regions of the two proteins that are not identical, further suggesting that regions where insulin I differs from insulin II become targets of autoimmunity owing to lack of expression in the thymus (TABLE 1) [56]. This is evident in particular for the B9–23 and B24–C36 epitopes (TABLE 1). Amino acid differences between the two proteins might also play a role in inducing T-cell responses to the C-peptide [69]. Moreover, the transgenic expression of *Ins2* in the thymus and peripheral lymphoid tissues results in recessive tolerance and inhibits diabetes development, but responses to insulin I still exist and diabetes is not suppressed completely [74]. Thus, while two models have been generated that express *Ins2* in the thymus, both resulting in inhibition of diabetes development [71,74], the critical experiment, expressing *Ins1* in the thymus, has not been carried out.

Insulin mutant mice

As discussed, the B9–23 insulin epitope is a major target T-cell epitope in NOD mice. Mutating the B9–23 peptide by substituting the native tyrosine at B16 with alanine abrogates T-cell reactivity of insulin autoreactive NOD T-cell clones [75]. Based on these findings, Nakayama and colleagues created NOD-*Ins1/Ins2* knockouts that expressed a mutated insulin transgene carrying alanine at B16 [76]. Since the mutated (pro)insulin has preserved biological activity, the introduction of the transgene prevents metabolic diabetes in mice lacking both native insulin genes, while at the same time removing the putative triggering epitope represented by the native B9–23 epitope. Thus, this mouse model allowed study of the role of the native insulin genes, and in particular the lack of an

Table 1. Amino acid sequences of mouse insulin II and I and of human insulin.

| B-chain                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | C-peptide | A-chain |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|---------|
| Mouse Insulin II                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |           |         |
| FVKQHLCG <b>SHLVEALYLVCGERG</b> FFYTP <b>MS</b> -RR-EV EDPQV <b>AQ</b> LELGGGPG <b>AG</b> DLQTLALEVA <b>QQ</b> -KR-GIVDQCCTSICSLYQLENYCN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |           |         |
| Mouse Insulin I                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |           |         |
| FVKQHLCG <b>PHLVEALYLVCGERG</b> FFYTP <b>KS</b> -RR-EV EDPQV <b>EQ</b> LELGGSPG _ _DLQTLALEVA <b>RQ</b> -KR-GIVDQCCTSICSLYQLENYCN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |           |         |
| Human Insulin                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |           |         |
| FVNQHLCG <b>SHLVEALYLVCGERG</b> FFYTP <b>KT</b> -RR-EAE DLQVGQVELGGGPGAGSLQPLALEGSLQ-KR-GIVEQCCTSICSLYQLENYCN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |           |         |
| Amino acid residues that differ between mouse insulin II and I are in bold. Note that the two mouse protein sequences differ at position 9 of the B chain, which is part of the insulin B-chain 9–23 T-cell epitope (underlined). This epitope is a target in both humans and mice. Another epitope targeted in both patients and nonobese diabetic mice encompasses the B-chain and C-peptide junction (B24–C36, shown in italics), with evidence for both CD4 and CD8 T-cell responses. Note that once again the two mouse proteins differ at position 29. Additional epitopes are reported in the region. The sequence of the human A-chain 1–15 CD4 T-cell epitope is also underlined, which is reportedly post-translationally modified. This region of the A-chain also contains an autoantibody epitope associated with disease development [8–13]. |           |         |

immunogenic B9–23 epitope, on the development of autoimmune diabetes. Female NOD mice bearing only the mutated insulin gene did not develop IAA, insulinitis or autoimmune diabetes, demonstrating that the B9–23 epitope is a critical and essential trigger for diabetes development. Importantly, the expression of the transgene in the thymus, which contains a mutated residue at B16, did not prevent diabetes development in mice that expressed one copy of *Ins1*, as diabetes was observed in most mice bearing just one *Ins1* copy [76,77]. This finding suggests that T cells specific for the native B9–23 epitope could not be negatively selected since *Ins1* is not transcribed in the thymus, while they were triggered in the periphery by the expression of insulin I in the pancreas (FIGURE 1). These observations further support the concept that NOD mice have defective tolerance to insulin I because only *Ins2* is expressed in the thymus, and that the presence of sequence differences at position B9 of the B9–23 epitope may trigger autoreactive T cells and diabetes development (FIGURE 1, TABLE 1).

Humans only have one insulin gene, and sequence differences such as those noted between the mouse insulin I and II proteins do not directly apply to human Type 1A diabetes. Although the molecular mechanisms underlying loss of tolerance to insulin appear to differ slightly in experimental mice and humans, the main epitopes targeted by anti-insulin immune responses appear remarkably similar (TABLE 1). Other factors (HLA restriction, processing of peptides and structural and biochemical features) may contribute to render these amino acid sequences particularly susceptible to become targets of autoimmunity. Moreover, qualitative differences in the expression of several autoantigens may be caused by alternative splicing in the target organ compared with the thymus, which in several cases were associated with autoimmunity [60]. There is evidence, for example, that the alternative splicing may influence the thymic expression of the islet molecules IA-2 [62] and IGRP [78]. Recent evidence suggests that alternative splicing may also affect (pro)insulin expression, at least in transfected cell lines, and that splicing may be regulated by *INS* polymorphisms [79], which were already shown to affect transcription of the native, full-length *INS* mRNA in the thymus. Diabetes-predisposing *INS* haplotypes appear to influence the expression of alternatively spliced transcripts, one of which lacks the sequences encoding the recently identified insulin A1–15 T-cell epitope [48]. Since full-length transcripts were detected previously in the human thymus [65], further investigations should examine whether the alternative splice transcripts are detected in the thymus, specifically in thymic epithelial cells and DCs, the relationship with genetic polymorphism and the potential impact of the spliced transcripts on the generation of CD4 T cells against the A1–15 epitope.

Mouse models involving manipulation of other autoantigens

Studies have been performed to test whether the other autoantigens also have an essential or triggering role in autoimmune diabetes. NOD-IA-2 knockout mice develop diabetes similar to nontransgenic littermates, indicating that autoimmunity to

IA-2 is not necessary for diabetes development in NOD mice [80]. Moreover, thymic expression of GAD did not alter diabetes development and in some studies it accelerated diabetes [81,82], while NOD-GAD knockout mice developed diabetes at normal rates [83]. Overall, insulin is the only autoantigen for which genetic manipulation in rigorous mouse models has shown consistent and dramatic effects on diabetes development. Of the three major autoantigens, insulin is the only one that clearly appears essential for diabetes development in the mouse.

### Insulin as a therapeutic agent for autoimmunity in diabetes

Additional support for a critical role of (pro)insulin in diabetes development derives from experimental and clinical studies in which proinsulin, insulin or peptides were used to modulate autoimmunity and delay or prevent diabetes. In NOD mice, both subcutaneous and oral insulin can delay or prevent diabetes; oral insulin induces regulatory CD4 T cells [84–87]. Naso-respiratory insulin has also been shown to protect NOD mice from diabetes [88]. T-cell responses to insulin were no longer detected and there was an increased production of regulatory cytokines, such as interleukin (IL)-4 and IL-10, associated with increased IAA titers. The protective effect was explained by the induction of suppressor CD8  $\gamma\delta$  T cells. In addition, the intranasal administration of the proinsulin peptide B24–C36 results in the stimulation of CD4 regulatory cells and prevents diabetes in NOD mice [89]. Moreover, both the intranasal or subcutaneous administration of the B9–23 peptide protected NOD mice from diabetes development [90]. Similarly, a mutated B9–23 peptide (altered peptide ligand) was shown to have immunomodulatory properties, delayed diabetes and reduced its incidence in NOD mice [91]. It is also important to note that careful selection of therapeutic peptides should take into consideration their anaphylactic potential, as lethal reactions have been reported in mice treated with the B9–23 peptide [92] or GAD peptides [93]. Moreover, peptides should be chosen based on their binding to selected HLA molecules, for example, to avoid binding to class I molecules, as this may in turn elicit cytotoxic CD8 T-cell responses, which may promote diabetogenesis [89], responses that are likely to be stimulated by mucosal exposure to antigen [94].

At the clinical level, a pilot study conducted in the early 1990s reported a reduced incidence of diabetes development in autoantibody-positive first-degree relatives who were treated with small doses of subcutaneous insulin [95]. This study was followed by one of the largest prevention trials ever performed in first-degree relatives, the Diabetes Prevention Trial – Type 1 (DPT-1), which screened over 100,000 relatives [96]. The study had two arms, in which the effects of parenteral insulin or oral insulin were evaluated in subjects identified through ICA and IAA screening. The parenteral study was a randomized, controlled, nonblinded clinical trial. Of the approximately 3000 ICA-positive relatives identified, 2103 underwent further genetic, immunological and metabolic staging to quantify their risk. More than 300 had a projected 5-year risk greater than

50% and they were randomized to either close observation or an intervention that consisted of low-dose subcutaneous ultra-lente insulin, administered twice daily for a total dose of 0.25 units per kilogram of bodyweight per day, plus an annual 4-day continuous intravenous infusion of insulin. With a median follow-up of 3.7 years, the study concluded that insulin at the dosage used in this study did not delay or prevent progression to overt diabetes. It is important to recognize that the dose of insulin used in DPT-1 was much lower than the dose that was reported to be effective in NOD mice [97].

The other arm of DPT-1 was a randomized, double-masked, placebo-controlled clinical trial to test whether oral insulin administration could delay or prevent disease in nondiabetic first-degree relatives at risk of diabetes [98]. The study involved 372 subjects with a 5-year risk projection of 26–50%. They were randomized to oral insulin (7.5 mg/day) or placebo. The annualized rate of diabetes development was similar in both groups: 6.4% with oral insulin and 8.2% with placebo. However, the analysis of a subgroup of relatives ( $n = 263$ ) with IAA levels more than 80 nU/ml demonstrated a statistical difference between the two groups. The annualized rate of diabetes in the treated group was 6.2%, compared with 10.4% in the placebo group ( $p = 0.015$ ). While the main conclusion from the trial was that oral insulin did not delay or prevent diabetes, the difference observed in the subgroup of relatives with confirmed IAA over 80 nU/ml suggests that oral insulin could have beneficial effects. It is particularly important that such an effect of oral insulin was seen in subjects with IAA, thus showing a link between the putative effects of the insulin treatment and autoimmunity to insulin. Based on the concept of oral tolerance [99], oral insulin administration may induce immune regulation and have an effect on insulin-specific immune responses and progression to diabetes, a hypothesis that will be tested in an upcoming clinical trial being currently designed by the Type 1 Diabetes TrialNet study group.

A study from Japan in patients diagnosed with latent autoimmune diabetes of the adult (LADA) and confirmed by GAD autoantibody positivity, reported that low doses of subcutaneous insulin may preserve C-peptide secretion and prevent or delay progression to insulin dependence [100], while C-peptide loss and insulin-dependence was observed more often in patients treated with a sulfonylurea. The results of this study may be interpreted as suggesting a protective effect of low dose insulin therapy on progression of islet autoimmunity in LADA patients. However, one must be cautious because, in this study, doses of insulin were adjusted in each patient to maintain very good standards of care (fasting plasma glucose  $<120$  mg/dl, HbA<sub>1c</sub>  $<7\%$ , and postprandial hyperglycemia  $<200$  mg/dl) and the paper did not specify the insulin doses used. Given that the patients in this study had diabetes, the study obviously lacked a control group that received no treatment; however, it is difficult to ascertain whether the difference observed may be due to a protective effect of insulin, directly or indirectly influencing the immune system and/or  $\beta$ -cell function and survival, or to a iatrogenic effect of sulfonylurea treatment in patients with a slowly progressive form of autoimmune diabetes.

Based on the hypothesis that exposure to a relevant antigen through the mucosal interface would overall promote immune regulation [101], a pilot trial in children at risk of developing Type 1 diabetes demonstrated a beneficial effect from the intranasal administration of insulin [102]. This randomized, crossover study showed that children with a first-phase insulin release greater than the first percentile experienced no further deterioration of insulin secretion over a median follow-up of 3 years. Moreover, intranasal insulin was associated with decreased T-cell responses to insulin. The results of this pilot study are consistent with the induction of immune regulation and inhibition of insulin-specific cellular responses. Similarly to the related studies in NOD mice [88], elevations in IAA titers were noted, which are possibly a marker of T helper (Th)2 deviation. This study has set the stage for a larger clinical trial to further explore the efficacy of intranasal insulin (INIT II) currently in its launching phase. Additional trials to test the safety and efficacy of insulin peptides are ongoing. A Phase I trial in which a modified insulin B9–23 peptide (altered peptide ligand) was administered subcutaneously to normal individuals and new onset patients has shown no major safety concerns [103]. It also appears that the altered peptide induced a Th2 bias, which may inhibit T-cell responses, while data on overall efficacy are not presently available.

Future clinical trials should also evaluate a number of critical factors, including the nature of the antigen or peptide, the dose, the route/mode of administration, genetic factors and the stage of disease at which the subjects enter the trial, as these can all influence efficacy and safety. Considering that the safety profile of insulin is well characterized and that insulin is approved by the US FDA for clinical use, insulin itself is presently the most promising compound for inclusion in antigen-based strategies to prevent autoimmune diabetes. Future trials are likely to explore the safety and efficacy of insulin or peptides in combination with immunomodulatory agents, possibly building on the positive results of the trials in which a monoclonal antibody to CD3 was used to immunomodulate T-cell responses and preserve  $\beta$ -cell function at diabetes onset [104,105]. Because of the multiplicity and heterogeneity of the autoimmune responses associated with Type 1A diabetes development, antigen-based therapies that target other autoantigens may also be effective, perhaps depending on the predominant response in a patient at the time of identification and the HLA type. There are ongoing trials evaluating the potential efficacy of antigen-based therapies involving the administration of GAD or a heat-shock protein peptide [106]. Strategies involving the administration of multiple antigens may perhaps be very effective and could be tested once the individual safety and efficacy of each antigen formulation is established. Eventually, complementary approaches aimed at selectively eliminating autoreactive T cells, re-establishing tolerance to multiple islet antigens and preserving and regenerating  $\beta$ -cell mass may be required to most effectively antagonize islet autoimmunity and reverse its consequences.

### Expert commentary

There has been much debate over which of several autoantigens targeted in Type 1A diabetes is essential for disease development and triggers  $\beta$ -cell autoimmunity. The evidence reviewed in this article, obtained from the study of patients and experimental mouse models, supports the concept that insulin is such an autoantigen, certainly in mouse models. It is also very likely that, in most patients, insulin may be the antigen that is targeted first, although further studies in humans to better characterize islet autoimmunity and T-cell responses in the pancreas are needed. We should consider that there is significant individual heterogeneity of the immune responses and temporal fluctuations of autoreactive T cells. However, there is more than sufficient evidence to justify the pursuit of clinical studies to test the potential of insulin as a therapeutic agent to antagonize autoimmunity in Type 1A diabetes. Based on the results of the DPT-1 Oral Insulin trial, evidence of ongoing insulin autoimmunity should be used to identify subjects with the highest likelihood of a clinical benefit.

### Five-year view

During the next 5 years, further progress will be made in the characterization of target insulin epitopes and on the specificity and phenotype of autoreactive T cells. Furthermore, data will be accumulated on humoral and T-cell responses to insulin and/or specific insulin epitopes during the natural history of the disease, in relation to disease development and risk. Upcoming clinical trials will test the safety and efficacy of insulin and/or insulin peptides and various routes of administration, in preventing and/or delaying disease onset in individuals with ongoing  $\beta$ -cell autoimmunity (secondary prevention). Further studies will combine the administration of insulin and/or insulin peptides together with drugs that would synergistically affect specific immune functions to increase therapeutic efficacy. Full results from these important studies are likely to become available within the next 7–10 years.

Assuming that significant safety and efficacy are shown, further trials may then be conducted in subjects who are genetically at risk but lack evidence of ongoing autoimmunity (primary prevention).

### Information resources

Websites with useful information about Type 1 diabetes, ongoing research efforts and consortia, clinical trials and funding agencies are listed below:

- Online book chapters on Type 1 diabetes are freely accessible and updated periodically on the web page of the Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center  
[www.uchsc.edu/misc/diabetes/books.html](http://www.uchsc.edu/misc/diabetes/books.html)
- Type 1 Diabetes TrialNet  
[www.diabetestrialnet.org](http://www.diabetestrialnet.org)
- Intranasal Insulin Trial II (INIT II)  
[www.diabetestrials.org/initii.html](http://www.diabetestrials.org/initii.html)
- Type 1 Diabetes Genetics Consortium (T1DGC)  
[www.t1dgc.org](http://www.t1dgc.org)
- The MHC database (dbMHC; includes a Type 1A diabetes site with HLA data)  
[www.ncbi.nlm.nih.gov/projects/mhc](http://www.ncbi.nlm.nih.gov/projects/mhc)
- American Diabetes Association (ADA)  
<http://diabetes.org>
- Juvenile Diabetes Research Foundation (JDRF)  
[www.jdrf.org](http://www.jdrf.org)
- Diabetes Research Institute, University of Miami  
[www.diabetesresearch.org](http://www.diabetesresearch.org)
- Diabetes Clinical Research page at the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK)  
[www.niddk.nih.gov/patient/patientdiabetes.htm](http://www.niddk.nih.gov/patient/patientdiabetes.htm)

### Key issues

- Insulin is a critical autoantigen in autoimmune diabetes, as demonstrated by studies in mouse models that involve genetic manipulation of the insulin genes. There is strong evidence that insulin is also a critical autoantigen in human Type 1A diabetes. However, autoimmunity against pancreatic  $\beta$ -cells is heterogeneous. Not all patients may have autoimmune responses against the same autoantigens or epitopes, and autoimmune responses, especially T-cell responses, may not necessarily be present at the same time but might rather be triggered in several temporal waves, complicating the identification of such responses and the ability to define their relationship to disease development.
- Data from the Diabetes Prevention Trial – Type 1 suggests that oral insulin may delay and/or prevent overt diabetes. Further clinical trials are being started or designed to further test the efficacy of insulin in preventing the development of diabetes in autoantibody-positive first-degree relatives.
- There is a clear need to obtain robust data on whether peripheral blood T-cell reactivity correlates well with disease progression in the pancreas, and how and whether changes in the frequency and phenotype of autoreactive T cells in peripheral blood reflect changes in disease activity. To this end, it is critical to develop coordinated efforts to facilitate access to pancreata from patients and prediabetic subjects, identified through the autoantibody screening of pancreas donors and pancreas transplant recipients with persistent or recurrent autoimmunity.

# Conflict of interest

The author is a member of the Type 1 Diabetes TrialNet, a network of clinical centers, experts in diabetes and immunology, and specialized laboratories dedicated to testing new approaches to understanding and preventing Type 1A diabetes. This clinical research program seeks to prevent Type 1A diabetes in high-risk people and to preserve insulin production in those newly diagnosed. Type 1 Diabetes TrialNet is supported by the National

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