Insulin and type 1 diabetes: immune connections
Sloboda Culina1,2, Vedran Brezar1,2 and Roberto Mallone1,2,3
1INSERM, U986, DeAR Lab Avenir, Saint Vincent de Paul Hospital, 82 Avenue Denfert Rochereau, 75674 Paris Cedex 14, France, 2Paris Descartes University, Sorbonne Paris Cité, Faculté de Médecine, Paris, France and 3Assistance Publique Hôpitaux de Paris, Service de Diabétologie, Hôtel Dieu, Paris, France
(Correspondence should be addressed to R Mallone at INSERM, U986, DeAR Lab Avenir, Saint Vincent de Paul Hospital; Email: roberto.mallone@inserm.fr)

Abstract
Insulin is the hormone produced by pancreatic β-cells, with a central role in carbohydrate and fat metabolism. Together with its precursors preproinsulin and proinsulin, insulin is also a key target antigen (Ag) of the autoimmune islet destruction leading to type 1 diabetes. Being recognized by both autoantibodies (aAbs) and autoreactive T cells, insulin plays a triggering role, at least in rodent models, in diabetes pathogenesis. It is expressed not only by β-cells but also in the thymus, where it plays a major role in central tolerance mechanisms. We will summarize current knowledge concerning insulin, its role in β-cell autoimmunity as initial target Ag, its recognition by aAbs and autoreactive T cells, and the detection of these immune responses to provide biomarkers for clinical trials employing insulin as an immune modulatory agent.

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Introduction
Type 1 diabetes (T1D) is one of the most common autoimmune diseases, resulting from the destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. At the crossroads between endocrine and immune pathways, insulin is the central actor in this disease. It is the key hormone of glucose metabolism, which is lacking in T1D. At the same time, it is one of the key molecular target antigens (Ags) recognized by autoreactive T cells, which are responsible for the destruction of β-cells leading to the metabolic derangements observed in T1D.

From a historical standpoint, T1D was not recognized as an autoimmune disease until the 70s. It was in 1965 that Gepts (1) described pancreatic islet inflammation (insulitis) as a hallmark of recent-onset T1D. This observation was followed 10 years later by the discovery of autoantibodies (aAbs) by Bottazzo et al. (2), who showed that sera of T1D patients were capable of recognizing pancreatic islet sections. These aAbs became the first biomarkers of T1D and attempts to attribute them a pathogenic role monopolized the attention of scientists for several years. Although unsuccessful, these attempts led to the molecular identification of aAb targets, and insulin was the first to be identified (3). The role of T lymphocytes rather than aAbs was subsequently suggested in the late 70s by Huang et al. (4), demonstrating the cytotoxic activity of T cells from T1D patients on insulinoma cells. The availability of the nonobese diabetic (NOD) mouse model in the 80s helped to definitely establish the role of T lymphocytes in T1D pathogenesis and to identify insulin as a major target not only for aAb but also for autoreactive T cells.

Although insulin was the first target Ag described, several other self-Ags were found to be recognized by B and T lymphocytes and associated with T1D (5). Besides being expressed by β-cells, mainly in subcellular compartments like secretory granules, most of these self-Ags are also expressed by cells that are not damaged by the autoimmune reaction. This suggests that a defective interaction between target islets and immune effector cells and the interplay between metabolic and immune pathways may be critical in T1D development (6). In this regard, insulin may play a leading role, standing at the crossroad of these pathways.

Insulin
Insulin is member of a family of peptide hormones characterized in more than 100 vertebrate species. It is a globular protein comprising an A and a B chain, which are composed of 21 and 30 residues respectively. These two chains are linked covalently by two interchain disulfide bonds (between amino acids A7 and B7 and A20 and B19) and one intrachain disulfide...
Insights into the relationship between PI and β-cell autoimmunity. The thymus is the organ where central tolerance takes place, i.e. where T lymphocytes are ‘educated’ to avoid β-cell Ags to DCs and presentation to T cells for their activation. On the contrary, the subsequent events are well documented (Fig. 2B), lying on a solid ground of experimental data obtained in animal models. In order to be activated and acquire their pathogenic role, T cells that escape central tolerance mechanisms in the thymus encounter β-cell Ags in the periphery, more precisely in the pancreatic lymph nodes (PLNs). β-Cell Ag release triggered by poorly defined mechanisms – perhaps linked to physiological waves of β-cell apoptosis during tissue remodeling (7) – leads to Ag uptake by Ag-presenting cells (APCs) like DCs, which ferry these Ags to PLNs. If autoreactive T lymphocytes capable of recognizing these Ags circulate through PLNs, they stop, undergo activation, and expand. They subsequently exit lymph nodes to reach pancreatic islets and mediate β-cell destruction (Fig. 2B). Experiments in NOD mice support the importance of this first Ag encounter, as early removal of PLNs (at 3 weeks of age) protects mice from diabetes development, which is not the case when PLNs are removed at a later time point (10 weeks of age) (8).

**PPI as the initiating β-cell Ag**

Several key Ags of β-cell autoimmunity have been identified as molecular targets of human autoreactive T cells and/or aAbs: PI, glutamic acid decarboxylase (GAD), tyrosine phosphatase-like islet cell Ag 2 (IA-2), zinc transporter 8 (ZnT8), and islet glucose-6-phosphatase catalytic subunit-related protein (IGRP). In autoimmunity, a popular tenet known as epitope spreading holds that one primary self-Ag initiates pathogenesis, and tissue destruction through targeting of this Ag further releases other ones, thus perpetrating the autoimmune cascade. PI has been identified as the initiating β-cell Ag in the NOD mouse.

**β-Cell autoimmunity**

T1D is a complex disease that involves many different immune players (both innate and adaptive), a susceptible genetic background and elusive environmental factors. Insights into T1D pathogenesis have been offered by animal models that spontaneously develop diabetes in the absence of any experimental trigger, a rather unique feature in the autoimmunity field. One such model is the Bio-Breeding (BB) rat, which develops diabetes with the same incidence in males and females, with a peak between 9 and 18 weeks of age. The second, most widely used model is the NOD mouse, which develops diabetes between weeks 12 and 30 with a stronger penetrance in females (~90 vs ~40% in males). In parallel, other models that require induction of diabetes were also generated and are broadly used. They include transgenic mice and/or models where viral infections trigger β-cell autoimmunity. In most of these models, a foreign viral Ag is transgenically expressed in β-cells under the control of the rat insulin promoter, so that diabetes can be rapidly induced by virus infection or Ag immunization, usually under conditions that promote inflammation and/or dendritic cell (DC) maturation. These models, even if not perfect, represent extremely valuable tools in the T1D research field.

The initiating events in T1D pathogenesis are yet to be elucidated. The importance of genetic background (mainly protective or susceptible HLA alleles), central and peripheral tolerance, and unknown environmental factors is well documented (Fig. 2A) but does not clarify the triggering event(s) leading to the initial provision of β-cell Ags to DCs and presentation to T cells for their activation. On the contrary, the subsequent events are well documented (Fig. 2B), lying on a solid ground of experimental data obtained in animal models. In order to be activated and acquire their pathogenic role, T cells that escape central tolerance mechanisms in the thymus encounter β-cell Ags in the periphery, more precisely in the pancreatic lymph nodes (PLNs). β-Cell Ag release triggered by poorly defined mechanisms – perhaps linked to physiological waves of β-cell apoptosis during tissue remodeling (7) – leads to Ag uptake by Ag-presenting cells (APCs) like DCs, which ferry these Ags to PLNs. If autoreactive T lymphocytes capable of recognizing these Ags circulate through PLNs, they stop, undergo activation, and expand. They subsequently exit lymph nodes to reach pancreatic islets and mediate β-cell destruction (Fig. 2B). Experiments in NOD mice support the importance of this first Ag encounter, as early removal of PLNs (at 3 weeks of age) protects mice from diabetes development, which is not the case when PLNs are removed at a later time point (10 weeks of age) (8).

**Figure 1** Amino acid sequence of preproinsulin (PPI). Amino acid sequence of human PPI leader sequence, B chain, C-peptide, and A chain is compared with mouse PPI1 (amino acid differences designated in red) and PPI2 (amino acid differences designated in blue). Disulphide bridges are designated with -S-S- symbols. Amino acid numbering is given both with respect to the proinsulin sequence alone (amino acids B1–A21, in black) and to the complete PPI sequence (amino acid 1–110, in red).
Figure 2 β-cell autoimmunity. (A) Pancreatic insulin-producing β cells undergo apoptosis following poorly defined triggers. These triggers are well documented in terms of genetic predisposition (susceptible HLA alleles), but much less so for environmental factors (infections?). They contribute to different degrees to (I) rupture of central tolerance, which allows migration of autoreactive T cells toward the periphery. (II) Local inflammation, with recruitment of innate immune cells such as neutrophils, macrophages, and NK cells in the pancreas, secretion of inflammatory cytokines, and other molecules (e.g., NO, oxidative radicals and chemokines) that (III) lead to β-cell apoptosis. (B, IV) Apoptotic β cells make antigens (Ags) such as insulin available for uptaking by immature dendritic cells (DCs). (V) On their way to pancreatic lymph nodes (PLNs), these DCs mature due to inflammatory stimuli and (VI) encounter naïve T cells (Tn). (VII) Autoreactive Tn escaped from thymic selection which recognize these Ag-loaded mature DCs are activated, differentiate into effector T cells (Teff), undergo clonal expansion, and (VIII) leave PLNs to migrate to the pancreas. (IX) Here, they perform their cytotoxic activities, leading to β-cell destruction and T1D.

self-recognition. The main thymic subset that endogenously expresses insulin is composed of medullary thymic epithelial cells (mTECs) (9, 10), and this ectopic expression is regulated by the Aire gene (11, 12). High-affinity recognition of insulin on the surface of mTECs by developing T lymphocytes (also called thymocytes) leads to an apoptotic death signal known as negative selection. When thymic insulin expression is defective (see below), accelerated diabetes develops (13). This may be due either to the escape of insulin-reactive
T cells toward the periphery in the absence of negative selection (14) and/or to defective positive selection of insulin-reactive regulatory T cells (Tregs) (15). Other cell populations in the thymus could also play a role in central tolerance. As recently described by Hadeiba et al. (16), plasmacytoid DCs can contribute to this process through C-C chemokine receptor 9-dependent ferrying of peripheral Ags to the thymus, resulting in subsequent deletion of Ag-reactive thymocytes. The relevance of this process in T1D pathogenesis is yet to be elucidated. The recent description of other cell populations capable of ectopically expressing tissue-specific Ags in peripheral lymph nodes under the control of Aire (17) adds one further level of complexity to our understanding of how the autoimmune potential can go unleashed.

The importance of insulin in murine T1D development is supported by three seminal studies: i) insulin knockout (Ins−/−) NOD mice. Contrary to humans, rodents express two isoforms of insulin: insulin 1 (Ins1), which is mainly expressed in the islets, and Ins2, which is the prevalent isomorph in the thymus. Ins2−/− NOD mice, which lack most insulin expression in the thymus, display accelerated T1D (13), thus lending support to the importance of central tolerance in T1D pathogenesis. Conversely, Ins1−/− NOD mice are less susceptible to T1D (18). ii) Nakayama et al. (19) generated NOD mice in which endogenous Ins1 and Ins2 genes were deleted and replaced with a hormonally active Ins transgene bearing a single amino acid mutation at position B16. This mutation completely protected these mice from T1D and insulinis, due to disrupted insulin recognition by T cells. iii) Finally, Krishnamurthy et al. (20) further supported the initiating role of PI by showing that NOD mice rendered tolerant to insulin by transgenically overexpressing it in APCs are not only protected from T1D but also do not develop responses to the immunodominant IGRP206-214 epitope, suggesting that these responses lay downstream of insulin-specific ones. On the contrary, mice rendered tolerant to IGRP were not protected from T1D, confirming the downstream and dispensable role of this Ag in the pathogenic cascade.

Although this body of data in the NOD mouse points to insulin as the triggering Ag of T1D, a similar role in human pathogenesis remains unsettled. Owing to insulin as the triggering Ag of T1D, a similar role of this Ag in the pathogenic cascade.

The presence of serum anti-insulin aAbs (IAAs) in newly diagnosed T1D subjects was first demonstrated by Palmer et al. (3) in 1983. Identification of other aAb specificities such as GAD (5), IA-2 (23), insulin precursor PI (24), and ZnT8 (25) followed. Rapid development of biochemical aAb assays validated in international T1D Antibody Workshops (26, 27) was achieved due to the identification and molecular cloning of these islet Ags.

Together with other β-cell aAbs, IAAs are used in clinical practice for etiological classification of new-onset diabetes cases (autoimmune vs nonautoimmune) and for T1D risk stratification in susceptible individuals. In T1D subjects younger than 15 years, IAA prevalence is particularly high (54%), while the prevalence of at least one among GAD, IA-2, and IAA aAbs is 97% (28). In young adults, 15- to 34-year-olds, the frequency of each of these aAbs is in the range of 40–60%, and at least one of the specificities is present in 80% of these patients (29). IAA prevalence displays an inverse relationship with age: 81% before age 10 and 61% between 10 and 20 years (30). This higher IAA prevalence in T1D children compared with adults further suggests that diabetes pathogenesis and autoimmune progression may differ depending on age.

Despite the fact that β-cell destruction is mediated by T cells, aAbs are excellent disease markers and their appearance precedes the clinical onset of T1D (31). No single aAb specificity alone performs well at predicting disease, and the positive predictive value increases with the number of positive aAbs, but not with their titers (32, 33, 34). No unique pattern stems out in the order of appearance of these aAbs, although IAAs often appear first (but are frequently absent in adults), whereas GAD, IA-2, and ZnT8 aAbs appear around the same time. The practical implication of this notion is that multiple aAb screening is necessary to achieve suitable predictive values of T1D risk such as those required for prevention trials.

IAA detection could also inform decisions for recruiting subjects into insulin-based clinical trials, as
suggested by Mamchak et al. (35). These authors treated NOD mice with a combined therapy (CT) comprising a short-course anti-CD3 mAb treatment and oral insulin therapy. In order to identify mice most likely to benefit from CT, they used a validated mathematical model of murine T1D pathophysiology (T1D PhysioLab Platform) (36) for defining suitable biomarkers that could differentiate responders from nonresponders and that are translatable into human trials. They showed that animals that best responded to this CT had elevated IAAs before therapy (35). These results are consistent with human data obtained in a post hoc analysis of the Diabetes Prevention Trial of T1D (DPT-1) (37), showing that patients with the highest IAA titers responded better (i.e. slower decline in residual insulin secretion) following oral insulin treatment.

Even though aAbs are not involved in T1D pathogenesis, several studies in NOD mice highlighted a role for aAb-secreting B lymphocytes. NOD mice rendered deficient in B lymphocytes, either by treatment with depleting mAbs (38) or by genetic knockout of the Ig μ chain (NOD.Igμ null mice) (39), are highly resistant to diabetes. Furthermore, the B-cell-depleting mAb rituximab showed not only a beneficial effect on diabetes protection and reversal in NOD mice (40) but also on β-cell preservation in new-onset T1D patients (41). However, this role of B lymphocytes is not linked to aAb secretion but to their function as APCs (42). Indeed, NOD mice harboring B lymphocytes deficient for major histocompatibility complex (MHC) class II expression remain strongly protected from diabetes, despite the intact ability of DCs and macrophages to express MHC class II and to present Ags (43). This ability of B lymphocytes to act as diabetogenic APCs is directly related to their Ag specificity, as increasing the frequency of insulin-specific B-cell clones significantly accelerated diabetes development in NOD mice (44).

**T-cell responses to insulin**

Like any other Ag, PI is also presented to T lymphocytes in the form of short peptide fragments (also called epitopes), in the context of either HLA class I (for CD8 +) or class II molecules (for CD4 + T cells). This presentation can take place on the surface of the β-cell itself (for HLA class I only, at least in the human) or of APCs such as DCs, macrophages, and B lymphocytes (for both HLA class I and II). Pancreatic islets are rich in resident DCs, and the pathways by which these DCs process and present PI show some unique features. Indeed, Calderon et al. (45) has shown that these DCs are capable of uptaking molecules not only from dying β-cells but also from insulin granules exocytosed by viable β cells. The first implication of these observations is that β-cell death may not be a requirement for initial priming of insulin-reactive T cells. Secondly, insulin granules were shown to contain not only (pro)insulin but also catabolic byproducts in the form of free PI peptides, notably PI B9–23 (46). Fine analysis of PI-specific CD4 + T cells in the NOD mouse further revealed two patterns of reactivity: ‘type A’, i.e. CD4 + T cells that respond to DCs pulsed either with PI B9–23 peptide or with the whole insulin protein and are highly deleted in the thymus; not ‘type B’, i.e. CD4 + T cells that respond only to the PI B9–23 peptide exogenously added to DCs or to freshly isolated intra-islet DCs endogenously presenting PI B9–23 uptaken from insulin granules. These unique features of intra-islet insulin presentation further explain why thymic negative selection is so permissive toward insulin-specific T cells. This is because whole insulin processing by thymic DCs preferentially generates a PI B13–21 epitope, which binds the HLA class II molecule IAg7 with high affinity (47). This leads to efficient negative selection of type A CD4 + T cells (Fig. 3). Conversely, PI B9–23 processing by intra-islet DCs gives rise to a PI B12–20 epitope, which binds IAg7 with much lower affinity. Thus, type B T cells recognizing PI B12–20 efficiently escape negative selection, as they do not get exposed to this epitope in the thymus. Instead, they get promptly activated in the islets, where PI B12–20 can be processed from the PI B12–23 peptide released from β-cell granules, at high enough concentrations to bypass the low IAg7 binding affinity (47) (Fig. 3). It is possible that similar islet-specific Ag processing pathways may rule T-cell activation against other β-cell Ags present in secretory granules, such as the recently described chromogranin A (48).

The central pathogenic role of T lymphocytes in T1D is well documented in animal models using different experimental approaches: i) the efficient transfer of diabetes by T cells from diabetic or prediabetic animals into naïve recipients (49, 50, 51); ii) the prevention of diabetes by injection of mAbs that interfere with T-lymphocyte activation; or iii) with Ag presentation to T cells; and iv) the absence of diabetes in diabetes-prone mice in which genes controlling T-lymphocyte differentiation or activation are invalidated (52). The same concept is supported in humans by observations of a predominant infiltration of T lymphocytes and IFNγ + cells (mostly CD8 + T lymphocytes) within the islets of prediabetic or T1D patients (53, 54, 55).

Despite this knowledge, the clinical applications of T-cell biomarkers remain in their infancy (56). Large efforts are ongoing to develop and standardize T-cell assays among different laboratories worldwide, coordinated by the T-Cell Workshop of the Immunology of Diabetes Society (57, 58). This lag is due to the technical challenges of T-cell assays that, contrary to the biochemical aAb assays, require live cells and exquisite sensitivity, thus exposing them to the risk of spurious results, as even minute contaminants can lead to nonspecific responses (59). We could estimate PI-reactive CD8 + T cells detected by IFNγ enzyme-linked immunospot (ELISpot) to be present at a median frequency of 0.004% (range, 0.0008–0.08%) of
PBMCs (60), which exemplifies the difficulties in detecting these cells. Additional hurdles include a large interindividual variation in the $\beta$-cell epitopes recognized, depending on HLA haplotypes, age (61), disease duration (62), calling for a continued effort to identify relevant Ags and epitopes. Indeed, target epitopes are different between T1D children and adults (61) and IFN$\gamma$ responses elicited by them are short-lived, as they wane already 6 months after diagnosis. Moreover, the preferred target epitopes change along the course of disease, gradually focusing on previously subdominant IA-2 and IGRP epitopes (62). This shift also applied to different epitopes derived from PI. Indeed, new-onset T1D patients preferentially displayed T-cell reactivities against the leader sequence epitope PPI6–14, whereas the B-chain epitope PI33–42 was predominantly recognized in long-standing patients (63).

For many years, investigations focused on CD4$^+$ T lymphocytes, while CD8$^+$ T cells moved into the spotlight only recently. This historical bias was due to the long-known association between diabetes and HLA class II predisposing and protective alleles. The recent recognition that HLA class I alleles can also modulate T1D risk (61, 64, 65) has further emphasized the role of CD8$^+$ T cells. More recently, a direct association between these T cells and $\beta$-cell destruction has been suggested in humans. Coppieters et al. (66) used frozen pancreas sections from 45 cadaveric T1D donors with disease duration ranging from 1 week to > 50 years (in comparison with 27 nondiabetic controls) for a systematic analysis of insulin content. CD8$^+$ insulitic lesions, HLA class I hyperexpression, and the specificity of CD8$^+$ T cells for six defined islet Ags, using in situ HLA class I tetramer staining. These tetramer experiments indicate that CD8$^+$ T cells found within insulitic lesions recognize known islet Ags, hinting to a direct link between T-cell autoimmunity and $\beta$-cell destruction. Interestingly, islets from patients close to diagnosis usually displayed one single specificity of islet-reactive CD8$^+$ T cells, whereas multiple specificities were commonly present with long-standing disease (66). The predominant specificity of these CD8$^+$ cells was for insulin epitopes. A more direct demonstration of the pathogenic potential of human CD8$^+$ T cells has been provided by the group of Peakman (67). A CD8$^+$ T-cell clone recognizing a HLA-A2-restricted PPI 15–24 epitope was capable of lysing primary human HLA-A2 islets. Interestingly, this cytotoxic activity was enhanced by increasing glucose concentrations, due to increased presentation of this epitope on the surface of $\beta$-cells. This latter observation points to the importance of metabolic variables in conditioning $\beta$-cell vulnerability to autoimmune destruction. A similar cytotoxic activity capable of killing human islets was reported for CD8$^+$ T-cell clones recognizing a HLA-A24-restricted PPI15–24 epitope (68).

The characterization of islet-specific Tregs has been more troublesome, as their frequency is probably
lower than for islet-specific effector T cells and their *in vitro* expansion potential more difficult to trigger. Arif et al. (69) suggested that islet-specific T cells with a regulatory-like phenotype (IL10-secreting) may also be present in both T1D and healthy individuals. These authors demonstrated the presence of both pro-inflammatory (IFN-γ-producing) and regulatory (IL10-producing) CD4+ T cells recognizing PI and IA-2 epitopes. While pro-inflammatory cells were predominantly detected in T1D patients, IL10-secreting ones were preferentially found in healthy subjects. Furthermore, T1D patients displaying higher IL10 responses were characterized by an older age at T1D onset, suggesting that these regulatory responses may keep autoimmunity in check for some time (69). This report was recently echoed by our observations on PI-specific CD8+ T cells, showing that these cells could be rarely detected in healthy subjects but, when found, they displayed an IL10+ phenotype not observed in T1D patients, suggestive of a regulatory bias (63). The group of Harrison (70) further reported that PI- and GAD-specific CD4+ Treg clones can be obtained *in vitro* under standard stimulating conditions in the absence of any exogenous cytokines. Similarly, GAD- and IGRP-specific CD4+ Treg clones were obtained by *in vitro* stimulation of FoxP3-negative CD4+ T cells (71). Whether such clones are only generated or can also be isolated *ex vivo* and their mechanism(s) of suppression remain to be investigated.

### Insulin in clinical trials

As insulin was the first and remains the most studied T1D Ag, it was also the first islet Ag employed in mouse studies and subsequently translated into clinical trials. The rationale of insulin-based T1D clinical trials is twofold: i) to restore insulin-specific immune tolerance; and/or ii) to put the β-cell ‘at rest’ when providing hormonally active exogenous insulin s.c., thus relieving the workload on the endogenous insulin secretory capacity. Numerous preclinical studies in NOD mice (72, 73, 74, 75, 76, 77, 78, 79) provided promising results that, once translated into human trials, enticed disappointment. There are a number of reasons (Table 1) (80, 81) that could explain this failure (82): i) the phylogenetic difference between the human and murine immune system; ii) differences in diabetogenic process – NOD mice are kept in homogenous conditions both with regard to the genetic background (constant inbreeding through brother–sister mating) and to the environment (specific pathogen-free animal facilities); iii) contrary to NOD mice, there is no conclusive evidence that insulin has an initiating role in human T1D; iv) the timing of treatment is different, as clinical trials are performed at a time when the first signs of β-cell autoimmunity (i.e. aAbs) are already present and is likely that at this stage Ag spreading has already occurred; v) the dose employed in human trials with s.c. insulin is not equivalent to that used in NOD preclinical models; and vi) duration of treatment. We recently showed (83) the relevance of these two latter points by treating 3-week-old NOD mice with a protocol more similar to the one employed in s.c. insulin trials. First, treatment was only for 7 weeks, shorter than the lifelong regimen employed in previous mouse studies. Secondly, s.c. insulin was used at two different doses: the high dose (0.5 U/mouse) used in previous mouse studies and the low dose (0.005 U/mouse) equivalent to that used in human trials. Indeed, the insulin dose previously administered in animal studies was ~200-fold higher than that later used in humans (84). The results obtained demonstrate that neither insulin regimen yielded any durable clinical protection or immune modification with this short-term treatment. Only the high dose achieved some effects: it delayed but did not prevent diabetes onset, it reduced insulitis, and suppressed C-peptide secretion. However, all these effects were only transient and rapidly lost upon

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**Table 1** Comparison of autoimmune diabetes in NOD mice and humans.

<table>
<thead>
<tr>
<th>Features</th>
<th>NOD mice</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetics</td>
<td>Inbred strain (genetically identical animals)</td>
<td>Unique individuals (genetically diverse)</td>
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<td>Environment</td>
<td>Uniform SPF environment</td>
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<td>Immune system</td>
<td>10–25% circulating neutrophils</td>
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<td>Insulin genes</td>
<td>No MHC class II expression on T cells</td>
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<td>Incidence</td>
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<td>Maternal aAbs</td>
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<td>Probably protective</td>
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<td>Insulin as initiating Ag</td>
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<tr>
<td>B cells required</td>
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<td>Probably not</td>
</tr>
<tr>
<td>Treatment timing</td>
<td>Before onset of β-cell autoimmunity</td>
<td>After onset of β-cell autoimmunity (aAb +)</td>
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SPF, specific pathogen-free; aAb, autoantibody.
<table>
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<tr>
<th>Antigen type</th>
<th>Antigen</th>
<th>Formulation</th>
<th>Route</th>
<th>Trial (phase)</th>
<th>Subjects</th>
<th>Outcome and/or immune biomarkers</th>
<th>Reference</th>
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<td>Protein</td>
<td>Insulin</td>
<td>Short-acting insulin</td>
<td>I.v.</td>
<td>I.v. insulin (phase I)</td>
<td>Recent-onset T1D</td>
<td>Higher stimulated C peptide and lower HbA1c vs s.c. NPH insulin</td>
<td>(87)</td>
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<td>Protein</td>
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<td>S.c. + i.v.</td>
<td>Joslin insulin prophylaxis trial (phase I)</td>
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<td>Suggestive of efficacy</td>
<td>(88)</td>
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<td>(84)</td>
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<td>ORALE (phase II)</td>
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<td>(91)</td>
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<td>Oral insulin tolerance</td>
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<tr>
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<td>Insulin</td>
<td>Short-acting insulin</td>
<td>Oral</td>
<td>NIH/ADA/JDRF oral insulin (phase III)</td>
<td>At risk IAA⁺</td>
<td>Increase in aAb and decrease in T-cell proliferative responses to insulin</td>
<td>(94)</td>
</tr>
<tr>
<td>Protein</td>
<td>Insulin</td>
<td>Short-acting insulin</td>
<td>Intranasal</td>
<td>INIT-I (phase I)</td>
<td>At risk IAA⁺</td>
<td>No effect; decrease in IFNγ T-cell responses to PI; and decrease in Ab responses to exogenous insulin</td>
<td>(95)</td>
</tr>
<tr>
<td>Protein</td>
<td>Insulin</td>
<td>Short-acting insulin</td>
<td>Intranasal</td>
<td>DIPP (phase III)</td>
<td>At risk</td>
<td>Recent-onset, noninsulin-dependent T1D</td>
<td>(96)</td>
</tr>
<tr>
<td>Protein</td>
<td>Insulin</td>
<td>Short-acting insulin</td>
<td>Intranasal</td>
<td>Intranasal insulin in T1D patients (phase II)</td>
<td>At risk</td>
<td>Ongoing</td>
<td>(101)</td>
</tr>
<tr>
<td>Protein</td>
<td>Insulin</td>
<td>Short-acting insulin</td>
<td>Intranasal</td>
<td>INIT-II (phase II)</td>
<td>At risk with preserved first-phase insulin response</td>
<td>Planned</td>
<td>(97)</td>
</tr>
<tr>
<td>Peptide</td>
<td>Insulin</td>
<td>B chain in incomplete Freund’s adjuvant</td>
<td>Oral or intranasal</td>
<td>Pre-POINT</td>
<td>Recent-onset IAA⁺ T1D</td>
<td>Increased TGFβ1 production</td>
<td>(98)</td>
</tr>
<tr>
<td>Peptide</td>
<td>Proinsulin</td>
<td>PlC19-A3</td>
<td>Intradermal</td>
<td>IBC-VS01 (phase I)</td>
<td>Long-standing T1D</td>
<td>Transient PI-specific IL10 secretion in 3/18 patients at 30 μg</td>
<td>(99)</td>
</tr>
<tr>
<td>Modified peptide</td>
<td>Insulin</td>
<td>NBI-6024 (B₉₋₂₃ APL)</td>
<td>S.c.</td>
<td>NBI-6024-0003 (phase I)</td>
<td>Recent-onset T1D</td>
<td>Shift from Th1 to Th2 responses</td>
<td>(100)</td>
</tr>
<tr>
<td>Modified peptide</td>
<td>Insulin</td>
<td>NBI-6024 (B₉₋₂₃ APL)</td>
<td>S.c.</td>
<td>Neurocrine NBI-6024 (phase II)</td>
<td>Recent-onset T1D</td>
<td>No effect</td>
<td>(101)</td>
</tr>
<tr>
<td>Modified protein DNA plasmid</td>
<td>Insulin</td>
<td>Insulin-coupled ECDI-fixed autologous leukocytes</td>
<td>I.v.</td>
<td>ITN insulin-coupled leukocytes</td>
<td>At risk</td>
<td>Planned</td>
<td>(102)</td>
</tr>
<tr>
<td>Modified protein DNA plasmid</td>
<td>Proinsulin</td>
<td>BHT-3021 (PI plasmid)</td>
<td>I.m.</td>
<td>Bayhill BHT-3021 (phase I)</td>
<td>Recent-onset T1D</td>
<td>Completed</td>
<td>(103)</td>
</tr>
</tbody>
</table>
treatment discontinuation. The results of this ‘reverse translation’ from human to mouse exemplify possible reasons for unsuccessful trials (83).

The clinical trials performed to date, either preventative (in at-risk subjects) or interventional (i.e. in already diabetic subjects), using insulin as an immune modulatory agent are summarized in Table 2 and have been discussed in detail elsewhere (6, 82). The take-home message is that none of the strategies attempted so far has withheld the challenge of phase II–III trials showing significant clinical benefit. However, there have been trials reporting biomarker modifications suggestive of immune efficacy. For example, our T-cell immune monitoring studies conducted on slowly progressing adult T1D patients treated with intranasal insulin or placebo (85) show that PI-specific IFNγ responses are efficiently blunted by the active treatment. This effect is PI specific, as it is not observed for control recall Ags, and is accompanied by lower raises in Ab titers upon s.c. insulin challenge in vivo. These results suggest that immune modifications are efficiently induced but do not result in significant clinical benefit, as the decline in C-peptide secretion is not altered. It could be that this intervention is performed too late, at a time when most β-cells are already destroyed and the autoimmune reaction has already diversified far beyond insulin. Earlier intervention strategies using a combination of β-cell Ags may achieve better results by rescuing larger islet numbers and by impacting a larger repertoire of autoimmune T cells. Another critical point may be to more extensively use immune biomarkers to guide enrollment for Ag-specific immune intervention. As demonstrated in the oral insulin arm of the DPT-1 trial, at-risk subjects with higher IAA titers displayed some protection from T1D development (86). One likely interpretation of these results is that subjects with the most active immune responses against a given β-cell Ag are the ones most likely to benefit from tolerogenic vaccinations with this same Ag. Trial enrollment based on their immune reactivity could therefore significantly improve the risk-to-benefit balance.

Conclusion

Although definite evidence is lacking, the early appearance of IAA and the T1D susceptibility conferred by INS VNTR polymorphisms strongly suggest that insulin plays a central role in both mouse and human β-cell autoimmunity. Recent evidence shows that its role as a hormone abundantly secreted by β-cells may also influence its processing and presentation by DCs leading to autoimmune T-cell activation. First, metabolic stress may enhance presentation of insulin epitopes on the surface of β cells. Secondly, the extraordinary secretory activity of β cells may provide DCs with an exclusive, islet-specific source of insulin epitopes not available in lymphoid organs, notably in the thymus. These findings have implications for designing future clinical trials aimed at exploiting insulin as an immune modulatory agent. If insulin plays an initiating role in triggering the autoimmune cascade, early treatment is critical. Primary prevention strategies could be attempted in genetically at-risk individuals (i.e. first-degree relatives of T1D patients harboring T1D susceptibility alleles) before aAb appearance. Interventions aimed at relieving metabolic stress from the β cells may further reduce presentation of pathogenic epitopes. Lastly, the notion that insulin epitopes selectively derived from intra-islet processing of peptide byproducts released by β-cell granules provide a preferential pathway for activation of pathogenic (type B) T cells may invite the use of selected insulin peptides rather than whole (pro)insulin protein for vaccination strategies aimed at restoring immune tolerance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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