REVIEW

MECHANISMS IN ENDOCRINOLOGY

Insulin and type 1 diabetes: immune connections

Sloboda Culina^{1,2}, Vedran Brezar^{1,2} and Roberto Mallone^{1,2,3}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.

European Journal of Endocrinology 168 R19–R31

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

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).

bond (between amino acids A6 and A11) (Fig. 1). Transcription of the insulin gene results in the synthesis of preproinsulin (PPI), which contains an N-terminal 24-amino acid leader sequence. Once translocated into the endoplasmic reticulum (ER), the leader sequence is rapidly cleaved to generate proinsulin (PI). In the ER, PI is reduced and unfolded, then oxidized (disulfide pairing), and folded to yield a PI polypeptide composed of an insulin-like core (A and B chains) and a 35-amino acid connecting peptide (C-peptide), which connects the C-terminus of the B chain to the N-terminus of the A chain. Proteolytic cleavage of PI into insulin and the 31-amino acid C-peptide is initiated once PI moves to the Golgi and is completed in secretory vesicles. Insulin is protected from misfolding through a zinc-stabilized assembly, which leads to formation of insulin hexamers organized around two or four zinc atoms, stored in secretory granules. Upon secretion into the portal circulation, insulin hexamers dissociate into bioactive insulin monomers.

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).

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.

Insights into the relationship between PI and autoimmune progression can be gained by considering the mechanisms of central immune tolerance. The thymus is the organ where central tolerance takes place, i.e. where T lymphocytes are 'educated' to avoid

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 uptake 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 isoform 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 insulinitis, 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 IGRP_{206–214} 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 the phenomenon of epitope spreading, responses to a triggering Ag (presumably PI) can be rapidly overgrown by secondary responses to other β-cell targets. This poses a difficult challenge in human T1D, since at the time when aAbs, the first signs of β-cell autoimmunity, become detectable, the autoimmune response is already floridly ongoing. Nonetheless, anti-insulin aAbs (IAA) are the earliest ones to appear, especially in children, suggesting a role for this Ag in the early steps of disease progression. Additional Ags such as GAD, IA-2, and ZnT8 have been identified as major aAb targets in humans, which is not the case in NOD mice, for which insulin remains the only aAb specificity identified to

date. Of further note, human *INS* variable number of tandem repeats (VNTR) polymorphisms are strongly associated with T1D susceptibility, conferring an odds ratio for disease development of ~2.5, which is second only to that conferred by the HLA class II *DQB1* locus (odds ratio ~6.8) (6). Similar to the phenotype observed in *Ins2*^{−/−} NOD mice, humans harboring *INS* VNTR susceptibility alleles display reduced PI expression in the thymus, thus leading to inefficient central tolerance (21, 22). The weight of *INS* VNTR polymorphisms on T1D susceptibility further hints to the importance of this Ag in triggering human β-cell autoimmunity.

aAb responses to insulin

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 lead 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.Ig μ^{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_{B9–23} 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 IFN γ + 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 IFN γ enzyme-linked immunospot (ELISpot) to be present at a median frequency of 0.004% (range, 0.0008–0.08%) of

Figure 3 T-cell responses to insulin. Two types of CD4⁺ T cells were recently described by Calderon *et al.* Type A CD4⁺ T cells are deleted in the thymus because they recognize a PI_{B13-21} epitope that binds to NOD mouse MHC class II with high affinity. Type B CD4⁺ T cells escape thymic negative selection because they undergo poor interaction with their cognate PI_{B12-20} epitope, which binds MHC class II with low affinity. Once in the islets of Langerhans, these type B T cells can recognize this epitope on dendritic cells (DC). This is because islet DC can uptake exocytosed insulin granules, which contain peptide byproducts like PI_{B9-23} that can be more efficiently processed into PI_{B12-20}.

PBMCs (60), which exemplifies the difficulties in detecting these cells. Additional hurdles include a large interindividual variation in the β -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 γ 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 β -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 β -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 PPI₁₅₋₂₄ 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 β -cells. This latter observation points to the importance of metabolic variables in conditioning β -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 PPI₃₋₁₁ 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 *in vitro* 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 $\sim$ 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 insulinitis, and suppressed C-peptide secretion. However, all these effects were only transient and rapidly lost upon

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

Features	NOD mice	Humans
Genetics	Inbred strain (genetically identical animals)	Unique individuals (genetically diverse)
Environment	Uniform SPF environment	Heterogeneous environment
	10–25% circulating neutrophils	50–70% circulating neutrophils
	75–90% circulating lymphocytes	30–50% circulating lymphocytes
Immune system	No MHC class II expression on T cells	MHC class II expression on T cells
Insulin genes	Two	One
T-cell-driven insulinitis	Severe	Mild
Incidence	>80%	0.25–0.40% (5–30% in genetically susceptible)
Gender bias	Females	No
Peri-insulinitis	Yes	No
Maternal aAbs	Probably diabetogenic	Probably protective
Insulin as initiating Ag	Established	Debated
B cells required	Yes	Probably not
Treatment timing	Before onset of β -cell autoimmunity	After onset of β -cell autoimmunity (aAb+)

SPF, specific pathogen-free; aAb, autoantibody.

Table 2 Clinical trials employing insulin as immunomodulatory agent.

Antigen type	Antigen	Formulation	Route	Trial (phase)	Subjects	Outcome and/or immune biomarkers	Reference
Protein	Insulin	Short-acting insulin	I.v.	I.v. insulin (phase I)	Recent-onset T1D	Higher stimulated C peptide and lower HbA1c vs s.c. NPH insulin	(87)
Protein	Insulin	(Ultra)lente/regular insulin (s.c.) + short-acting insulin (i.v.)	S.c. + i.v.	Joslin insulin prophylaxis trial (phase I)	At risk	Suggestive of efficacy	(88)
Protein	Insulin	Lente/short-acting insulin (s.c.) + short-acting insulin (i.v.)	S.c. + i.v.	Schwabing insulin prophylaxis trial (phase I)	At risk	Suggestive of efficacy	(89)
Protein	Insulin	Ultralente insulin (s.c.) + short-acting insulin (i.v.)	S.c. + i.v.	DPT-1 parenteral arm (phase II)	At risk	No effect	(84)
Protein	Insulin	Ultralente insulin	S.c.	EPSCIT (phase II)	At risk	No effect	(90)
Protein	Insulin	Short-acting insulin	Oral	ORALE (phase II)	Recent-onset T1D	No effect	(91)
Protein	Insulin	Short-acting insulin	Oral	IMDIAB VII (phase II)	Recent-onset T1D	No effect	(92)
Protein	Insulin	Short-acting insulin	Oral	DPT-1 oral arm (phase III)	At risk	Some efficacy in IAA ⁺ subjects	(86)
Protein	Insulin	Short-acting insulin	Oral	Oral insulin tolerance (Phase III)	Recent-onset T1D	1 mg improved C-peptide responses in older patients and 10 mg accelerated C-peptide decline in younger patients	(93)
Protein	Insulin	Short-acting insulin	Oral	NIH/ADA/JDRF oral insulin (phase III)	At risk IAA ⁺	Ongoing	NCT 00419562
Protein	Insulin	Short-acting insulin	Intranasal	INIT-I (phase I)	At risk IAA ⁺	Increase in aAb and decrease in T-cell proliferative responses to insulin	(94)
Protein	Insulin	Short-acting insulin	Intranasal	DIPP (phase III)	At risk	No effect	(95)
Protein	Insulin	Short-acting insulin	Intranasal	Intranasal insulin in T1D patients (phase II)	Recent-onset, noninsulin-dependent T1D	No effect; decrease in IFN γ T-cell responses to PI; and decrease in Ab responses to exogenous insulin	(85)
Protein	Insulin	Short-acting insulin	Intranasal	INIT-II (phase II)	At risk with preserved first-phase insulin response IAA ⁺ children at high genetic risk for T1D	Ongoing	NCT 00336674
Protein	Insulin	Short-acting insulin	Oral or intranasal	Pre-POINT	Recent-onset IAA ⁺ T1D	Planned	(96)
Peptide	Insulin	Insulin B chain in incomplete Freund's adjuvant	I.m.	IBC-VS01 (phase I)	Recent-onset IAA ⁺ T1D	Increased TGF β production	(97)
Peptide	Proinsulin	PL _{C19-A3}	Intradermal	PI peptide immunotherapy (phase I)	Long-standing T1D	Transient PI-specific IL10 secretion in 3/18 patients at 30 μ g	(98)
Modified peptide	Insulin	NBI-6024 (B ₉₋₂₃ APL)	S.c.	NBI-6024-0003 (phase I)	Recent-onset T1D	Shift from Th1 to Th2 responses	(99)
Modified peptide	Insulin	NBI-6024 (B ₉₋₂₃ APL)	S.c.	Neurocrine NBI-6024 (phase II)	Recent-onset T1D	No effect	(100)
Modified protein	Insulin	Insulin-coupled ECDI-fixed autologous leukocytes	I.v.	ITN insulin-coupled leukocytes	At risk	Planned	(101)
DNA plasmid	Proinsulin	BHT-3021 (PI plasmid)	I.m.	Bayhill BHT-3021 (phase I)	Recent-onset T1D	Completed	NCT 00453375

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 IFN γ 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.

Funding

This work was supported by the JDRF (grant number 1-2008-106); Aviesan (TODAY project), INSERM Avenir, EFSD-JDRF-Novo Nordisk Programme in Type 1 Diabetes Research 2009, ANR-Blanc Immunotolerins. V Brezar was recipient of a PhD fellowship from the Ile-de-France CODDIM.

Acknowledgements

Figures 1, 2, and 3 were created using Servier Medical Art.

References

- Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 1965 **14** 619–633.
- Bottazzo GF, Florin-Christensen A & Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* 1974 **2** 1279–1283. (doi:10.1016/S0140-6736(74)90140-8)
- Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK & Paquette TL. Insulin antibodies in insulin-dependent diabetes before insulin treatment. *Science* 1983 **222** 1337–1339. (doi:10.1126/science.6362005)
- Huang SW & Maclaren NK. Insulin-dependent diabetes: a disease of autoaggression. *Science* 1976 **192** 64–66. (doi:10.1126/science.769160)
- Atkinson MA & Maclaren NK. Islet cell autoantigens in insulin-dependent diabetes. *Journal of Clinical Investigation* 1993 **92** 1608–1616. (doi:10.1172/JCI116745)
- Brezar V, Carel JC, Boitard C & Mallone R. Beyond the hormone: insulin as an autoimmune target in type 1 diabetes. *Endocrine Reviews* 2011 **32** 623–669. (doi:10.1210/er.2011-0010)
- Mathis D, Vence L & Benoist C. β -Cell death during progression to diabetes. *Nature* 2001 **414** 792–798. (doi:10.1038/414792a)
- Gagnerault MC, Luan JJ, Lotton C & Lepault F. Pancreatic lymph nodes are required for priming of β cell reactive T cells in NOD mice. *Journal of Experimental Medicine* 2002 **196** 369–377. (doi:10.1084/jem.20011353)

- 9 Derbinski J, Schulte A, Kyewski B & Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nature Immunology* 2001 **2** 1032–1039. (doi:10.1038/ni723)
- 10 Gotter J, Brors B, Hergenbahn M & Kyewski B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. *Journal of Experimental Medicine* 2004 **199** 155–166. (doi:10.1084/jem.20031677)
- 11 Palumbo A, Falco P, Ambrosini MT, Petrucci MT, Musto P, Caravita T, Pregno P, Bertola A, Cavallo F, Ciccone G *et al.* Thalidomide plus dexamethasone is an effective salvage regimen for myeloma patients relapsing after autologous transplant. *European Journal of Haematology* 2005 **75** 391–395. (doi:10.1111/j.1600-0609.2005.00533.x)
- 12 Yano M, Kuroda N, Han H, Meguro-Horike M, Nishikawa Y, Kiyonari H, Maemura K, Yanagawa Y, Obata K, Takahashi S *et al.* Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *Journal of Experimental Medicine* 2008 **205** 2827–2838. (doi:10.1084/jem.20080046)
- 13 Thebault-Baumont K, Dubois-Laforgue D, Krief P, Briand JP, Halbout P, Vallon-Geoffroy K, Morin J, Laloux V, Lehuen A, Carel JC *et al.* Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. *Journal of Clinical Investigation* 2003 **111** 851–857. (doi:10.1172/JCI16584)
- 14 Faideau B, Lotton C, Lucas B, Tardivel I, Elliott JF, Boitard C & Carel JC. Tolerance to proinsulin-2 is due to radioresistant thymic cells. *Journal of Immunology* 2006 **177** 53–60.
- 15 Gagnerault MC, Lanvin O, Pasquier V, Garcia C, Damotte D, Lucas B & Lepault F. Autoimmunity during thymectomy-induced lymphopenia: role of thymus ablation and initial effector T cell activation timing in nonobese diabetic mice. *Journal of Immunology* 2009 **183** 4913–4920. (doi:10.4049/jimmunol.0901954)
- 16 Hadeiba H, Lahl K, Edalati A, Oderup C, Habtezion A, Pachynski R, Nguyen L, Ghodsi A, Adler S & Butcher EC. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity* 2012 **36** 438–450. (doi:10.1016/j.immuni.2012.01.017)
- 17 Metzger TC & Anderson MS. Control of central and peripheral tolerance by Aire. *Immunological Reviews* 2011 **241** 89–103. (doi:10.1111/j.1600-065X.2011.01008.x)
- 18 Moriyama H, Abiru N, Paronen J, Sikora K, Liu E, Miao D, Devendra D, Beilke J, Gianani R, Gill RG *et al.* Evidence for a primary islet autoantigen (preproinsulin 1) for insulinitis and diabetes in the nonobese diabetic mouse. *PNAS* 2003 **100** 10376–10381. (doi:10.1073/pnas.1834450100)
- 19 Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF *et al.* Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 2005 **435** 220–223. (doi:10.1038/nature03523)
- 20 Krishnamurthy B, Dudek NL, McKenzie MD, Purcell AW, Brooks AG, Gellert S, Colman PG, Harrison LC, Lew AM, Thomas HE *et al.* Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. *Journal of Clinical Investigation* 2006 **116** 3258–3265. (doi:10.1172/JCI29602)
- 21 Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST & Patel DD. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR–IDDM2 susceptibility locus for type 1 diabetes. *Nature Genetics* 1997 **15** 293–297. (doi:10.1038/ng0397-293)
- 22 Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E & Polychronakos C. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nature Genetics* 1997 **15** 289–292. (doi:10.1038/ng0397-289)
- 23 Bonifacio E, Lampasona V, Genovese S, Ferrari M & Bosi E. Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *Journal of Immunology* 1995 **155** 5419–5426.
- 24 Bohmer K, Keilacker H, Kuglin B, Hubinger A, Bertrams J, Gries FA & Kolb H. Proinsulin autoantibodies are more closely associated with type 1 (insulin-dependent) diabetes mellitus than insulin autoantibodies. *Diabetologia* 1991 **34** 830–834. (doi:10.1007/BF00408359)
- 25 Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW *et al.* The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *PNAS* 2007 **104** 17040–17045. (doi:10.1073/pnas.0705894104)
- 26 Bingley PJ, Bonifacio E & Mueller PW. Diabetes Antibody Standardization Program: first assay proficiency evaluation. *Diabetes* 2003 **52** 1128–1136. (doi:10.2337/diabetes.52.5.1128)
- 27 Torn C, Mueller PW, Schlosser M, Bonifacio E & Bingley PJ. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. *Diabetologia* 2008 **51** 846–852. (doi:10.1007/s00125-008-0967-2)
- 28 Sabbah E, Savola K, Kulmala P, Veijola R, Vahasalo P, Karjalainen J, Akerblom HK & Knip M. Diabetes-associated autoantibodies in relation to clinical characteristics and natural course in children with newly diagnosed type 1 diabetes. The Childhood Diabetes in Finland Study Group. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 1534–1539. (doi:10.1210/jc.84.5.1534)
- 29 Torn C, Landin-Olsson M, Lernmark A, Palmer JP, Arnqvist HJ, Blohme G, Lithner F, Littorin B, Nystrom L, Schersten B *et al.* Prognostic factors for the course of β cell function in autoimmune diabetes. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 4619–4623. (doi:10.1210/jc.85.12.4619)
- 30 Williams AJ, Norcross AJ, Dix RJ, Gillespie KM, Gale EA & Bingley PJ. The prevalence of insulin autoantibodies at the onset of type 1 diabetes is higher in males than females during adolescence. *Diabetologia* 2003 **46** 1354–1356. (doi:10.1007/s00125-003-1197-2)
- 31 Srikanta S, Ganda OP, Rabizadeh A, Soeldner JS & Eisenbarth GS. First-degree relatives of patients with type I diabetes mellitus. Islet-cell antibodies and abnormal insulin secretion. *New England Journal of Medicine* 1985 **313** 461–464. (doi:10.1056/NEJM198508223130801)
- 32 Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, Bottazzo GF & Gale EA. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 1994 **43** 1304–1310. (doi:10.2337/diabetes.43.11.1304)
- 33 Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Loppinen T, Dyrberg T, Akerblom HK & Knip M. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. *Journal of Clinical Investigation* 1998 **101** 327–336. (doi:10.1172/JCI19879)
- 34 Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, Chase HP & Eisenbarth GS. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996 **45** 926–933. (doi:10.2337/diabetes.45.7.926)
- 35 Mamchak AA, Manenkova Y, Leconet W, Zheng Y, Chan JR, Stokes CL, Shoda LK, von Herrath M & Bresson D. Preexisting autoantibodies predict efficacy of oral insulin to cure autoimmune diabetes in combination with anti-CD3. *Diabetes* 2012 **61** 1490–1499. (doi:10.2337/db11-1304)
- 36 Shoda L, Kreuwel H, Gadkar K, Zheng Y, Whiting C, Atkinson M, Bluestone J, Mathis D, Young D & Ramanujan S. The Type 1 Diabetes PhysioLab Platform: a validated physiologically based mathematical model of pathogenesis in the non-obese diabetic mouse. *Clinical and Experimental Immunology* 2010 **161** 250–267. (doi:10.1111/j.1365-2249.2010.04166)

- 37 Skyler JS. Update on worldwide efforts to prevent type 1 diabetes. *Annals of the New York Academy of Sciences* 2008 **1150** 190–196. (doi:10.1196/annals.1447.055)
- 38 Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF & Naji A. B-cells are required for the initiation of insulinitis and sialitis in nonobese diabetic mice. *Diabetes* 1997 **46** 941–946. (doi:10.2337/diabetes.46.6.941)
- 39 Serreze DV, Chapman HD, Varnum DS, Hanson MS, Reifsnnyder PC, Richard SD, Fleming SA, Leiter EH & Shultz LD. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new “speed congenic” stock of NOD.Ig mu null mice. *Journal of Experimental Medicine* 1996 **184** 2049–2053. (doi:10.1084/jem.184.5.2049)
- 40 Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, Shlomchik MJ & Wen L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *Journal of Clinical Investigation* 2007 **117** 3857–3867. (doi:10.1172/JCI32405)
- 41 Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, McGee PE, Moran AM *et al.* Rituximab, B-lymphocyte depletion, and preservation of β -cell function. *New England Journal of Medicine* 2009 **361** 2143–2152. (doi:10.1056/NEJMoa0904452)
- 42 Mallone R & Brezar V. To B or not to B: (anti)bodies of evidence on the crime scene of type 1 diabetes? *Diabetes* 2011 **60** 2020–2022. (doi:10.2337/db11-0700)
- 43 Noorchashm H, Lieu YK, Noorchashm N, Rostami SY, Greeley SA, Schlachterman A, Song HK, Noto LE, Jevnikar AM, Barker CF *et al.* I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet β cells of nonobese diabetic mice. *Journal of Immunology* 1999 **163** 743–750.
- 44 Hulbert C, Riseili B, Rojas M & Thomas JW. B cell specificity contributes to the outcome of diabetes in nonobese diabetic mice. *Journal of Immunology* 2001 **167** 5535–5538.
- 45 Calderon B, Suri A, Miller MJ & Unanue ER. Dendritic cells in islets of Langerhans constitutively present β cell-derived peptides bound to their class II MHC molecules. *PNAS* 2008 **105** 6121–6126. (doi:10.1073/pnas.0801973105)
- 46 Mohan JF, Levisetti MG, Calderon B, Herzog JW, Petzold SJ & Unanue ER. Unique autoreactive T cells recognize insulin peptides generated within the islets of Langerhans in autoimmune diabetes. *Nature Immunology* 2010 **11** 350–354. (doi:10.1038/ni.1850)
- 47 Mohan JF, Petzold SJ & Unanue ER. Register shifting of an insulin peptide–MHC complex allows diabetogenic T cells to escape thymic deletion. *Journal of Experimental Medicine* 2011 **208** 2375–2383. (doi:10.1084/jem.20111502)
- 48 Stadinski BD, Delong T, Reisdorff N, Reisdorff R, Powell RL, Armstrong M, Piganelli JD, Barbour G, Bradley B, Crawford F *et al.* Chromogranin A is an autoantigen in type 1 diabetes. *Nature Immunology* 2010 **11** 225–231. (doi:10.1038/ni.1844)
- 49 Bendelac A, Carnaud C, Boitard C & Bach JF. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *Journal of Experimental Medicine* 1987 **166** 823–832. (doi:10.1084/jem.166.4.823)
- 50 Nagata M, Santamaria P, Kawamura T, Utsugi T & Yoon JW. Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic β -cells in nonobese diabetic mice. *Journal of Immunology* 1994 **152** 2042–2050.
- 51 Peterson JD & Haskins K. Transfer of diabetes in the NOD-scid mouse by CD4 T-cell clones. Differential requirement for CD8 T-cells. *Diabetes* 1996 **45** 328–336. (doi:10.2337/diabetes.45.3.328)
- 52 Delovitch TL & Singh B. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 1997 **7** 727–738. (doi:10.1016/S1074-7613(00)80392-1)
- 53 Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG & Gamble DR. *In situ* characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulinitis. *New England Journal of Medicine* 1985 **313** 353–360. (doi:10.1056/NEJM19850803130604)
- 54 Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M *et al.* Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *Journal of Clinical Investigation* 1993 **92** 2313–2322. (doi:10.1172/JCI116835)
- 55 Willcox A, Richardson SJ, Bone AJ, Foulis AK & Morgan NG. Analysis of islet inflammation in human type 1 diabetes. *Clinical and Experimental Immunology* 2009 **155** 173–181. (doi:10.1111/j.1365-2249.2008.03860.x)
- 56 Culina S & Mallone R. Immune biomarkers in immunotherapeutic trials for type 1 diabetes: cui prodest? *Diabetes & Metabolism* 2012. In press. (doi:10.1016/j.diabet.2012.05.005)
- 57 Mallone R, Mannering SI, Brooks-Worrell BM, Durinovic-Bello I, Cilio CM, Wong FS & Schloot NC. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. *Clinical and Experimental Immunology* 2011 **163** 33–49. (doi:10.1111/j.1365-2249.2010.04272.x)
- 58 Schloot NC, Meierhoff G, Karlsson Faresjö M, Ott P, Putnam A, Lehmann P, Gottlieb P, Roep BO, Peakman M & Tree T. Comparison of cytokine ELISpot assay formats for the detection of islet antigen autoreactive T cells. Report of the third immunology of diabetes society T-cell workshop. *Journal of Autoimmunity* 2003 **21** 365–376. (doi:10.1016/S0896-8411(03)00111-2)
- 59 Brezar V, Culina S, Osterbye T, Guillonneau F, Chiappetta G, Verdier Y, Vinh J, Wong FS, Buus S & Mallone R. T cells recognizing a peptide contaminant undetectable by mass spectrometry. *PLoS ONE* 2011 **6** e28866. (doi:10.1371/journal.pone.0028866)
- 60 Martinuzzi E, Lemonnier FA, Boitard C & Mallone R. Measurement of CD8 T cell responses in human type 1 diabetes. *Annals of the New York Academy of Sciences* 2008 **1150** 61–67. (doi:10.1196/annals.1447.015)
- 61 Scotto M, Afonso G, Osterbye T, Larger E, Luce S, Raverdy C, Novelli G, Bruno G, Gonfroy-Leymarie C, Launay O *et al.* HLA-B7-restricted islet epitopes are differentially recognized in type 1 diabetic children and adults and form weak peptide–HLA complexes. *Diabetes* 2012 **61** 2546–2555. (doi:10.2337/db12-0136)
- 62 Martinuzzi E, Novelli G, Scotto M, Blancou P, Bach JM, Chaillous L, Bruno G, Chatenoud L, van Endert P & Mallone R. The frequency and immunodominance of islet-specific CD8+ T-cell responses change after type 1 diabetes diagnosis and treatment. *Diabetes* 2008 **57** 1312–1320. (doi:10.2337/db07-1594)
- 63 Luce S, Lemonnier F, Briand JP, Coste J, Lahlou N, Muller S, Larger E, Rocha B, Mallone R & Boitard C. Single insulin-specific CD8+ T cells show characteristic gene expression profiles in human type 1 diabetes. *Diabetes* 2011 **60** 3289–3299. (doi:10.2337/db11-0270)
- 64 Nejentsev S, Howson JM, Walker NM, Szeszkó J, Field SF, Stevens HE, Reynolds P, Hardy M, King E, Masters J *et al.* Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. *Nature* 2007 **450** 887–892. (doi:10.1038/nature06406)
- 65 Noble JA, Valdes AM, Varney MD, Carlson JA, Moonsamy P, Fear AL, Lane JA, Lavant E, Rappner R, Louey A *et al.* HLA class I and genetic susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium. *Diabetes* 2010 **59** 2972–2979. (doi:10.2337/db10-0699)
- 66 Coppieters KT, Dotta F, Amiran N, Campbell PD, Kay TW, Atkinson MA, Roep BO & von Herrath MG. Demonstration of

- islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients. *Journal of Experimental Medicine* 2012 **209** 51–60. (doi:10.1084/jem.20111187)
- 67 Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, Van-Krinks C, Zaremba A, Rackham C, Allen JS, Tree TI *et al.* CTLs are targeted to kill β cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. *Journal of Clinical Investigation* 2008 **118** 3390–3402. (doi:10.1172/JCI35449)
 - 68 Kronenberg D, Knight RR, Estorninho M, Ellis RJ, Kester MG, de Ru A, Eichmann M, Huang GC, Powrie J, Dayan CM *et al.* Circulating preproinsulin signal peptide-specific CD8 T cells restricted by the susceptibility molecule HLA-A24 are expanded at onset of type 1 diabetes and kill β -cells. *Diabetes* 2012 **61** 1752–1759. (doi:10.2337/db11-1520)
 - 69 Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO & Peakman M. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *Journal of Clinical Investigation* 2004 **113** 451–463. (doi:10.1172/JCI19585)
 - 70 Dromey JA, Lee BH, Yu H, Young HE, Thearle DJ, Jensen KP, Mannering SI & Harrison LC. Generation and expansion of regulatory human CD4(+) T-cell clones specific for pancreatic islet autoantigens. *Journal of Autoimmunity* 2011 **36** 47–55. (doi:10.1016/j.jaut.2010.10.005)
 - 71 Long SA, Walker MR, Rieck M, James E, Kwok WW, Sanda S, Pihoker C, Greenbaum C, Nepom GT & Buckner JH. Functional islet-specific Treg can be generated from CD4+CD25– T cells of healthy and type 1 diabetic subjects. *European Journal of Immunology* 2009 **39** 612–620. (doi:10.1002/eji.200838819)
 - 72 Alleva DG, Gaur A, Jin L, Wegmann D, Gottlieb PA, Pahuja A, Johnson EB, Motheral T, Putnam A, Crowe PD *et al.* Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9–23) peptide. *Diabetes* 2002 **51** 2126–2134. (doi:10.2337/diabetes.51.7.2126)
 - 73 Arakawa T, Yu J, Chong DK, Hough J, Engen PC & Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nature Biotechnology* 1998 **16** 934–938. (doi:10.1038/nbt1098-934)
 - 74 Atkinson MA, Maclaren NK & Luchetta R. Insulinitis and diabetes in NOD mice reduced by prophylactic insulin therapy. *Diabetes* 1990 **39** 933–937. (doi:10.2337/diabetes.39.8.933)
 - 75 Daniel D & Wegmann DR. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23). *PNAS* 1996 **93** 956–960. (doi:10.1073/pnas.93.2.956)
 - 76 Hutchings P & Cooke A. Protection from insulin dependent diabetes mellitus afforded by insulin antigens in incomplete Freund's adjuvant depends on route of administration. *Journal of Autoimmunity* 1998 **11** 127–130. (doi:10.1006/jaut.1997.0184)
 - 77 Karounos DG, Bryson JS & Cohen DA. Metabolically inactive insulin analog prevents type 1 diabetes in prediabetic NOD mice. *Journal of Clinical Investigation* 1997 **100** 1344–1348. (doi:10.1172/JCI19654)
 - 78 Muir A, Peck A, Clare-Salzler M, Song YH, Cornelius J, Luchetta R, Krischer J & Maclaren N. Insulin immunization of nonobese diabetic mice induces a protective insulinitis characterized by diminished intra-islet interferon- γ transcription. *Journal of Clinical Investigation* 1995 **95** 628–634. (doi:10.1172/JCI17707)
 - 79 Zhang ZJ, Davidson L, Eisenbarth G & Weiner HL. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *PNAS* 1991 **88** 10252–10256. (doi:10.1073/pnas.88.22.10252)
 - 80 Mestas J & Hughes CC. Of mice and not men: differences between mouse and human immunology. *Journal of Immunology* 2004 **172** 2731–2738.
 - 81 Roep BO, Atkinson M & von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. *Nature Reviews. Immunology* 2004 **4** 989–997. (doi:10.1038/nri1502)
 - 82 Culina S, Boitard C & Mallone R. Antigen-based immune therapeutics for type 1 diabetes: magic bullets or ordinary blanks? *Clinical & Developmental Immunology* 2011 **2011** 286248. (doi:10.1155/2011/286248)
 - 83 Brezar V, Culina S, Gagnerault MC & Mallone R. Short-term subcutaneous insulin treatment delays but does not prevent diabetes in NOD mice. *European Journal of Immunology* 2012 **42** 1553–1561. (doi:10.1002/eji.201242394)
 - 84 Diabetes Prevention Trial – Type 1 Diabetes Study G. Effects of insulin in relatives of patients with type 1 diabetes mellitus. *New England Journal of Medicine* 2002 **346** 1685–1691. (doi:10.1056/NEJMoa012350)
 - 85 Fourlanos S, Perry C, Gellert SA, Martinuzzi E, Mallone R, Butler J, Colman PG & Harrison LC. Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes. *Diabetes* 2011 **60** 1237–1245. (doi:10.2337/db10-1360)
 - 86 Skyler JS, Krischer JP, Wolfsdorf J, Cowie C, Palmer JP, Greenbaum C, Cuthbertson D, Raffkin-Mervis LE, Chase HP & Leschek E. Effects of oral insulin in relatives of patients with type 1 diabetes: the Diabetes Prevention Trial – Type 1. *Diabetes Care* 2005 **28** 1068–1076. (doi:10.2337/diacare.28.7.1630)
 - 87 Shah SC, Malone JI & Simpson NE. A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus. *New England Journal of Medicine* 1989 **320** 550–554. (doi:10.1056/NEJM198903023200902)
 - 88 Keller RJ, Eisenbarth GS & Jackson RA. Insulin prophylaxis in individuals at high risk of type I diabetes. *Lancet* 1993 **341** 927–928. (doi:10.1016/0140-6736(93)91215-8)
 - 89 Fuchtenbusch M, Rabl W, Grassl B, Bachmann W, Standl E & Ziegler AG. Delay of type I diabetes in high risk, first degree relatives by parenteral antigen administration: the Schwabing Insulin Prophylaxis Pilot Trial. *Diabetologia* 1998 **41** 536–541. (doi:10.1007/s001250050943)
 - 90 Carel JC, Landais P & Bougneres P. Therapy to prevent type 1 diabetes mellitus. *New England Journal of Medicine* 2002 **347** 1115–1116. (doi:10.1056/NEJM200210033471415)
 - 91 Chaillous L, Lefevre H, Thivolet C, Boitard C, Lahlou N, Atlan-Gepner C, Bouhanick B, Mogenet A, Nicolino M, Carel JC *et al.* Oral insulin administration and residual β -cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale Group. *Lancet* 2000 **356** 545–549. (doi:10.1016/S0140-6736(00)02579-4)
 - 92 Pozzilli P, Pitocco D, Visalli N, Cavallo MG, Buzzetti R, Crino A, Spera S, Suraci C, Multari G, Cervoni M *et al.* No effect of oral insulin on residual β -cell function in recent-onset type 1 diabetes (the IMDIAB VII). IMDIAB Group. *Diabetologia* 2000 **43** 1000–1004. (doi:10.1007/s001250051482)
 - 93 Ergun-Longmire B, Marker J, Zeidler A, Rapaport R, Raskin P, Bode B, Schatz D, Vargas A, Rogers D, Schwartz S *et al.* Oral insulin therapy to prevent progression of immune-mediated (type 1) diabetes. *Annals of the New York Academy of Sciences* 2004 **1029** 260–277. (doi:10.1196/annals.1309.057)
 - 94 Harrison LC, Honeyman MC, Steele CE, Stone NL, Sarugeri E, Bonifacio E, Couper JJ & Colman PG. Pancreatic β -cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes. *Diabetes Care* 2004 **27** 2348–2355. (doi:10.2337/diacare.27.10.2348)
 - 95 Nanto-Salonen K, Kupila A, Simell S, Siljander H, Salonsaari T, Hekkala A, Korhonen S, Erkkola R, Sipila JI, Haaavisto L *et al.* Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial. *Lancet* 2008 **372** 1746–1755. (doi:10.1016/S0140-6736(08)61309-4)

- 96 Achenbach P, Barker J & Bonifacio E. Modulating the natural history of type 1 diabetes in children at high genetic risk by mucosal insulin immunization. *Current Diabetes Reports* 2008 **8** 87–93. (doi:10.1007/s11892-008-0017-y)
- 97 Orban T, Farkas K, Jalahej H, Kis J, Treszl A, Falk B, Reijonen H, Wolfsdorf J, Ricker A, Matthews JB *et al.* Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy. *Journal of Autoimmunity* 2010 **34** 408–415. (doi:10.1016/j.jaut.2009.10.005)
- 98 Thrower SL, James L, Hall W, Green KM, Arif S, Allen JS, Van-Krinks C, Lozanoska-Ochser B, Marquesini L, Brown S *et al.* Proinsulin peptide immunotherapy in type 1 diabetes: report of a first-in-man phase I safety study. *Clinical and Experimental Immunology* 2009 **155** 156–165. (doi:10.1111/j.1365-2249.2008.03814.x)
- 99 Alleva DG, Maki RA, Putnam AL, Robinson JM, Kipnes MS, Dandona P, Marks JB, Simmons DL, Greenbaum CJ, Jimenez RG *et al.* Immunomodulation in type 1 diabetes by NBI-6024, an altered peptide ligand of the insulin B epitope. *Scandinavian Journal of Immunology* 2006 **63** 59–69. (doi:10.1111/j.1365-3083.2005.01705.x)
- 100 Walter M, Philotheou A, Bonnici F, Ziegler AG & Jimenez R. No effect of the altered peptide ligand NBI-6024 on β -cell residual function and insulin needs in new-onset type 1 diabetes. *Diabetes Care* 2009 **32** 2036–2040. (doi:10.2337/dc09-0449)
- 101 Luo X, Herold KC & Miller SD. Immunotherapy of type 1 diabetes: where are we and where should we be going? *Immunity* 2010 **32** 488–499. (doi:10.1016/j.immuni.2010.04.002)

Received 11 August 2012

Revised version received 20 September 2012

Accepted 12 October 2012