Quarterly Medical Review

Pancreatic islet autoimmunity

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Available online: 22 November 2012

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Summary

Type 1 diabetes (T1D) represents 10 to 15% of all forms of diabetes. Its incidence shows a consistent rise in all countries under survey. Evidence for autoimmunity in human T1D relies on the detection of insulin, of islet cell antibodies, of activated β-cell-specific T lymphocytes and on the association of T1D with a restricted set of class II major histocompatibility complex (MHC) alleles. However, mechanisms that initiate the failure of immune tolerance to β-cell autoantigens remain elusive in common forms of T1D. T1D commonly develop as a multifactorial disease in which environmental factors concur with a highly multigenic background. The disease is driven by the activation of T-lymphocytes against pancreatic β-cells. Several years elapse between initial triggering of the autoimmune response to β-cells, as evidenced by the appearance or islet cell autoantibodies, and the onset of clinical diabetes, defining a prediabetes stage. Active mechanisms hold back autoreactive effector T-cells in prediabetes, in particular a subset of CD4+ T-cells (Treg) and other regulatory T-cells, such as invariant NKT cells. There is evidence in experimental models that systemic or local infections can trigger autoimmune reactions to β-cells. However, epidemiological observations that have accumulated over years have failed to identify undisputable environmental factors that trigger T1D. Moreover, multiple environmental factors may intervene in the disease evolution and protective as well as triggering environmental factors may be involved. Available models also indicate that local signals within the islets are required for full-blown diabetes to develop. Many autoantigens that are expressed by β-cells but also by the other endocrine islet cells and by neurons are recognized by lymphocytes along the development of T1D. The immune image of β-cells is that of native components of the β-cell membrane, as seen by B-lymphocytes, and of fragments of intracellular β-cell proteins in the form of peptides loaded onto class I MHC molecules on the β-cell surface and class I and class II molecules onto professional antigen presenting cells. Given the key role of T lymphocytes in T1D, the cartography of autoantigen-derived peptides that are presented to class I-restricted CD8+ T-cells and class II-restricted CD4+ T-cells is of utmost importance and is a necessary step in the development of diagnostic T-cell assays and of immunotherapy of T1D.
Among the two major clinical forms of diabetes, type 1 diabetes (T1D) is viewed exclusively as an immune disease, while type 2 diabetes (T2D) is viewed as a metabolic disease. T1D results from the selective destruction of the islet β-cells that secrete insulin, the other islet endocrine cells being preserved. T2D is the consequence of a defective insulin response to increased insulin needs that result from insulin resistance, but evolve with time to progressive loss of insulin secretion. The respective part of a functional defect and that of a decrease in the β-cell mass remains an open issue in T2D.

The dramatic increase in the incidence of T2D, which represents up to 80% of all forms of diabetes, has been coined a world epidemics, following the increasing incidence of obesity. T1D only represents 10 to 15% of all forms of diabetes. However, it is another diabetes epidemics that is likely to become a public health challenge. T1D incidence differs between and within countries, with the highest numbers in northern regions and a consistent rise (3%-4%/year) in all countries under survey. In Finland, T1D incidence was 20/106/year in 1972 and is now well above 65/106/year, increasing linearly with time. Neither geographical variations nor the incidence rise can be explained by genome variations, leading to postulate that environmental factors are involved. The role of the environment is also supported by the low concordance rate (30-40%) in monozygotic twins and data from migrants. However, no single environmental factor has so far been identified.

T1D and T2D share common concerns [1], the more so since obesity and T2D engage an innate immune response. Obesity is associated with chronic inflammation that has direct implications on the pathophysiology of T2D. The close evolutionary links between immune and metabolic pathways set up the possibility that T1D and T2D are not as different as initially foreseen. Uncommon forms of diabetes, namely Latent Autoimmune Diabetes in Adults (LADA), have been considered as possible overlaps between T1D and T2D. The lack of accurate biological criteria for T2D diagnosis leaves the issue of overlapping forms of diabetes open.

It is only in T1D that innate immune activation leads to the activation of antigen-specific lymphocytes that are directly responsible for the destruction of β-cells. T1D is an autoimmune disease driven by the activation of lymphocytes against pancreatic β-cells. While the successive steps that control the activation of autoreactive lymphocytes have been extensively studied in animal models, the disease process remains ill-defined in man [2]. However, the predominant role of T-lymphocytes is characteristic of both mouse and human T1D [3]. T1D is a highly multigenic disease both in the mouse [4] and in man [5]. In man, T-lymphocytes, especially CD8+ T-cells, are predominant within insulin in most cases [6-13]. Occurrence of T1D in a patient deprived of B-lymphocytes further underscores the role of T lymphocytes [14]. T1D will be exclusively considered in this article. Autoimmune pancreatitis [15,16] will not be considered beyond a possible continuum with classical β-cell-targeted T1D.

Clinical issues

The early detection of autoantibodies in normoglycemic individuals who have ultimately developed T1D has led to the description of a long preclinical phase that precedes full-blown hyperglycemia [17]. The first detection of autoantibodies can occur early following birth. A second incidence peak may be seen around puberty and shows more heterogeneous autoantibody profiles than in early forms of T1D [17]. Another remarkable feature of T1D is the high recurrence level of autoimmunity in long-standing patients who have been treated with exogenous insulin for years [18], as seen in T1D recipients of an isograft from a discordant, non-diabetic, twin. Recurrence in recipients is accompanied by a largely predominant CD8+ T-cell islet infiltration. It thus seems that β-cell-specific T-lymphocytes maintain immune memory for years after T1D onset. However, differentiation patterns of autoreactive T-lymphocytes, once diabetes diagnosed, remain largely unknown.

From an immunological standpoint, islet cell autoantibodies are strongly associated with the development of T1D. In the absence of reliable T-cell assays, detection of autoantibodies in subjects at genetic risk of T1D has proved the best marker for autoimmunity, including in uncommon forms of diabetes, as in LADA [1]. Despite differences in the pattern of autoantibodies, in the specificity of autoreactive T-cells and in the level of insulin resistance, the issue of a common, or distinct, pathogenesis of T1D and LADA remains open. LADA, in which islet antibodies, the most often anti-GAD autoantibodies, are detected at diagnosis, but patients may remain non-insulin-dependent for years due to slowly progressive β-cell destruction. The choice of insulin as a first line treatment of LADA is still discussed.

**Glossary**

APCs antigen presenting cells
GAD glutamate decarboxylase
GFAP glial fibrillary acidic protein
IA-2 islet Antigen 2
IAA insulin autoantibodies
ICA islet cell autoantibody
iNKT invariant NKT cells
LADA latent Autoimmune Diabetes in Adults
LCMV lymphocoriomeningitis virus
MHC major histocompatibility complex
NOD non-obese diabetic
T1D type 1 diabetes
T2D type 2 diabetes
Treg regulatory T-cells
Fulminant T1D has been initially described in Japan. It is characterized by the rapid development of the hyperglycemic syndrome and of ketoacidosis and by low HbA1c values at onset. It differs from common forms of T1D by the absence of autoantibodies and of insulin but despite the presence of mononuclear infiltrates in the exocrine gland and the destruction of glucagon-cells along with that of β-cells. An elevation of serum pancreatic enzymes is often observed. Frequent flu-like symptoms at onset of diabetes have suggested the possibility of a viral infection as a triggering event, but no unique virus has been identified [19]. A recent model of fulminant T1D has been described in CD28−/− non-obese diabetic (NOD) mice which develop an accelerated form of T1D upon injection of TLR3 ligand, i.e. polyinosinic-polycytidylic acid. A destruction of α-cells is seen in addition to the destruction of β-cells, as well as exocrine tissue damage, in this model. The autoimmune genetic background of this model open the possibility that fulminant diabetes may be, at least in some cases, an exacerbated form of autoimmune T1D [20]. This would imply that the selectivity of β-cell destruction is a feature of common forms of T1D, but may not apply to all forms of the disease. In a large series of patients including T1D, altered exocrine functions have been reported, suggesting the possibility of exocrine lesions in some forms of T1D as in fulminant diabetes [21].

The detection of autoantibodies against β-cell autoantigens remains the only diagnostic marker for autoimmune in T1D in clinical practice. The appearance of autoantibodies is still considered the starting point in the disease process and autoantibody detection is a reliable biomarker in the clinical diagnosis of T1D autoimmunity. However, autoantibodies face limitations. While autoantibodies detected against more than two different target autoantigens - i.e. islet cell autoantibody (ICA) detected by indirect immunofluorescence on frozen sections of human blood group O pancreata, anti-glutamate decarboxylase (GAD) autoantibodies, anti-insulin autoantibodies, anti-islet Antigen 2 (IA-2) autoantibodies (or ICA512 or IA-2β or PHOGRIN) – are highly predictive of T1D in normoglycemic individuals, they do not predict time to onset of T1D. Moreover, detection of autoantibodies to ≤ 2 autoantigens is poorly predictive. However, despite the key pathogenic role of T-cells in T1D, there is currently no T-cell assay in use in clinical practice, due to intrinsic difficulties in measuring T-cell responses. T-cells specific for autoantigens are present at a very low frequency in blood (i.e. 0.1–0.001%) [17,22,23]. In a large study in which 549 first degree relatives of T1D patients with ICA values above 20 JDF Units, 159 developed diabetes. While 12% individuals with ICA alone developed diabetes, the detection in addition of IA-2A, anti-GAD or anti-insulin antibodies gave hazard ratios of 7.2, 13.9 and 7.3, respectively, in univariate analysis. The highest HLA susceptibility haplotype, DQ8 (DQB1*0302) provided a hazard ratio of 1.68, while the protective haplotype DQ6 (DQB1*0602) provided a hazard ratio of 0.3, 5 ICA-positive DQ6+ subjects developing T1D. Positivity for one autoantibody, two autoantibodies and three autoantibodies led to hazard ratios of 9.8, 29.9 and 67.9, respectively [23].

**Preclinical models for type 1 diabetes**

Animal models have been instrumental in contributing to our current understanding of T1D, especially models in which diabetes develops spontaneously such as the NOD mouse, an inbred strain which shows striking similarities with human diabetes. NOD mice develop diabetes from 12 weeks on, with a higher prevalence in females than in males. Early histological alterations at 3 weeks of age consist of swelling of endothelial cells within islet vessels and infiltration by dendritic cells and macrophages around some islets. A peri-insular infiltration by T-cells, mostly CD4+ T-cells, is seen secondarily. T-cells control the activation of the autoimmune reaction and mediate β-cell destruction. In the mouse, T1D is transferred into naive recipients by T-cells, is prevented by antibodies that target T-lymphocyte activation and fails to develop when key genes in T lymphocyte differentiation or activation are non-functional [3]. Diabetes is prevented by injection of monoclonal antibodies that target membrane molecules involved in T-cell interaction with antigen presenting cells or with T-cell signaling that follows antigen recognition. The role of stem cells, of defective negative selection of T-cells in the thymus as well as an imbalance between regulatory T-cell subsets and effector T-cells have been shown to contribute to the autoimmune phenotype in the NOD model [24,25]. The BB rat develops diabetes with the same incidence in males and females and a peak between 60 and 120 days of age. A key feature in BB rat diabetes is lymphopenia and the lack of a key regulatory T-cell subset that expresses RT6 [25]. With the advent of transgenesis in the mouse, many new models have been generated that allow testing the role of each successive step in immune tolerance to autoantigens or β-cell-targeted transgenes [26–28]. Some transgenic models have been developed in which infections can trigger an autoimmune reaction against β-cells [27,29].

**Type 1 diabetes pathogenesis**

Indirect evidence for autoimmunity in human T1D relies on the frequent occurrence of diabetes in association with other autoimmune diseases, in particular endocrine and gut autoimmune diseases, the detection of insulin, islet cell antibodies, T-cell activation against β-cell antigens and the association of diabetes with a restricted set of class II major histocompatibility complex (MHC) alleles. However, mechanisms that initiate the failure of immune tolerance to β-cell antigens remain elusive in common forms of T1D. T1D commonly develop as a multifactorial disease in which environmental factors concur with a highly multigenic susceptibility background to allow failure of immune tolerance to β-cells to develop.
Environmental factors

Since the experimental demonstration in the mid 1950’s that autoimmune diseases could be elicited by immunization against self-tissues or autoantigens in complete Freund’s adjuvant, the rationale pursued for years assimilated autoimmunity to immune responses to foreign antigens. A leading scheme thus postulates that an environmental factor fouls the immune system and triggers the activation and the expansion of autoreactive lymphocytes. In these experimental conditions, autoimmunity results from presentation of autoantigens by professional antigen presenting cells (APCs) to autoreactive T-cells. The presentation of autoantigens can occur at sites that are distant from target tissues [30].

There is evidence in experimental models that systemic or local infections can trigger autoimmune reactions to β-cells. Molecular mimicry between viral proteins and β-cell transgenes, bystander activation of autoreactive T-cells in transgenic mice expressing a biased T-cell repertoire [30–32] or disruption of Th1/Th2 balance, such as following infection by the Kilham virus in RT1u rats [33], have been reported to induce diabetes. There is no such strong evidence to support the role of infections in triggering T1D in man. There are anecdotal reports of coincidence of viral infections and onset of T1D, and evidence for an increased prevalence of virus-specific IgM antibodies or detection of viral RNA or DNA in recent-onset T1D patients [34]. Viruses that have been most frequently involved are rubella virus, cytomegalovirus, mumps virus and coxsackie virus B4. This does not provide direct evidence, however, that viruses initiate the failure of tolerance to β-cell antigens in human diabetes. With the exception of congenital rubella, no unique virus has been convincingly reported to trigger diabetes development. Bystander activation of autoreactive T-cells by superantigens has also been suggested to trigger T1D in the human, but direct evidence favoring this mechanism remains elusive [13]. The role of infectious agents in triggering autoimmunity through molecular mimicry or activation of T-cells by superantigens leads to postulate that autoimmunity arises against an autoantigen that is specifically expressed by cells that will be destroyed by the autoimmune reaction, unless a low sensitivity threshold to destruction predisposes a given tissue to selective destruction by the autoimmune reaction. The evidence that this is the case in T1D is disputed. Many autoantigens are targets of autoantibodies and T-cells in T1D, most of which are expressed by β-cells but also by other islet endocrine cells and by neurons that are not damaged by the autoimmune process in common forms of the disease.

Alternative hypotheses need to take into account key features of T1D. Pathological conditions that induce abnormal presentation of autoantigens by antigen presenting cells remain unknown. Animal models of spontaneous diabetes and epidemiological data in the human challenge the obligatory role of environmental factors in triggering diabetes development. No triggering factor has been evidenced in the NOD mouse. Diabetes prevalence varies from one NOD colony to another, depending on the environment and possibly on genetic drift from the original stock. However, most infectious agents have shown neutral or protective effects rather than induction of diabetes in the NOD mouse. The highest prevalence of insulitis and diabetes in NOD mice is observed in pathogen-free colonies. However, even in pathogen-free environments, all animals do not develop overt diabetes, independently of known environmental factors.

In the human, the early occurrence of autoantibodies to β-cell antigens, especially anti-insulin autoantibodies, in children from a diabetic mother or a diabetic father leads to postulate that environmental triggering factors, intervenes at most in fetal or early life [35]. However, this observation is intriguing in several respects. Once ICA have developed, the progression to diabetes is determined by the age of antibody appearance and by the diversity of the autoantigens recognized. Epidemiological observations that have accumulated over years have failed to identify undisputable environmental factors that trigger autoimmune diabetes [36]. This applies to children from a diabetic mother or a diabetic father despite the fact that they were followed up from birth. This is quite unexpected if one considers that the dominant hypothesis in autoimmunity is that, on a highly multigenic susceptibility background, an environmental factor is the triggering event that initiates the failure of immune tolerance to β-cells. This model is challenged by both the failure to identify a unique environmental factor as the triggering factor of T1D in the human and by the NOD model, raising the possibility that stochastic events influence diabetes development. It must also be underscored that multiple environmental factors may intervene in the disease evolution and that protective as well as triggering environmental factors may be involved. Available models also indicate that local signals within the islets are required for full-blown diabetes to develop. The islets and pancreatic draining lymph nodes are central in the initial activation of autoreactive T lymphocytes in the NOD mouse [37,38]. In human, the role of a genetic region that controls the transcription of the insulin gene also points to the islet as an important parameter in the development of T1D [39,40]. The role of local factors in the disease process is further underscored by the striking heterogeneity of the islet infiltrate within individual pancreases [11].

Genetic background

Both testing of candidate genes and genome scans have identified a long list of regions possibly involved in genetic susceptibility to T1D both in man and in the NOD mouse [5,41–43]. With the exception of MHC class II genes, the 5’ flanking region of the insulin gene, the CTLA4 gene and possibly the PTPN22 gene, most susceptibility genes within these regions remain unknown. The likelihood that multiple genes contribute
to susceptibility and that most of them have, individually, a weak effect has hampered the characterization of further contributing genes. T1D linkage to markers located on numerous chromosomes have been evidenced in NOD crosses, allowing an estimate of an almost unlimited number of genetic loci possibly carrying diabetes susceptibility genes. Moreover, congenic mice harboring non-NOD alleles at idd loci indicate that several clustered genes in a given locus contribute to disease susceptibility. In addition, the loci identified also vary depending on the breeding partner used for crosses and, several loci are detected by different crosses, while others are only detected in unique crosses. Resistance genes are mixed with susceptibility genes even on diabetes-prone backgrounds and may explain late development of diabetes in conventional NOD mouse. Further genetic analysis suggests that strong interactions between different diabetes susceptibility (idd) genes modulate diabetes development [4].

Most forms of autoimmunity start with the presentation of autoantigens in the form of short peptides to T-lymphocytes. Class II major histocompatibility molecules are the “molecular filters” that determine the array of antigenic peptides presented to T-lymphocytes. Expression of susceptibility class II variants is a prerequisite to the development of an immune reaction of a defined specificity, and class II genes appear as the strongest genetic determinants of autoimmunity, as best exemplified in T1D. Overall, the MHC locus contributes approximately 40% of genetic susceptibility to T1D. In the human, a strong association between T1D and a restricted set of class II MHC alleles (IDDM1) has been evidenced. Diabetes susceptibility alleles carry a serine, an alanine, or a valine residue in position 57 of the class II DQB (DQB1*0302 or DQ2/DQB1*0201 on DR4 and DR3 haplotypes, respectively). Class II alleles carrying an aspartic acid in DQB57 are neutral or confer dominant protection (DQ6/DQB1*0602). The presence of an aspartic acid at residue 57 allows establishing a hydrogen bond with Arg78 on the DQα chain, alters the peptide binding cleft of the class II molecule and modifies the sequence profile of peptides that are presented to T-cell clones [44]. Class II knocked out mice carrying the human DQ8 and DQ6 alleles along with a B7 transgene under the control of the rat insulin promoter bring definitive evidence for a direct role of class II genes in diabetes susceptibility [45]. The NOD class II I-A^b_97 allele carries a unique B chain that shows strong structural homology with human DQ8 susceptibility alleles. The clustering of organ-specific autoimmune diseases observed in human T1D is shared by the NOD mouse and the BB rat which develop, among other autoimmune disorders, sialitis and thyroiditis, respectively. Beyond inheritance of genes that predispose to general failure of self-tolerance on both backgrounds, susceptibility regions identified by analysis of crosses between the NOD and conventional mouse strains harbor genes that control the targeting of autoimmunity towards β-cells, among which MHC class II genes [2]. Class II susceptibility alleles are disease specific. Congenic NOD mice expressing I-A^d_94 or I-A^b_9 instead of I-A^b_97 remain diabetes-free but develop extensive thyroiditis, especially in the presence of increase dietary iodine, and destructive sialitis, respectively. In addition, non-class II genes and environmental factors also contribute to direct the autoimmune response towards a target tissue on autoimmune-prone backgrounds. B7.2/^−/NOD mice develop an autoimmune peripheral neuropathy instead of diabetes. AIRE/^−/^− NOD mice, which lack a major gene involved in thymic selection of T-cells, are protected from diabetes but develop lymphoid infiltrates in organs that are not damaged in the original NOD strain and destruction of acinar structures [46]. Infection by mycobacteria favors the development of a lupus phenotype in conventional NOD mice [2].

Considering the multiplicity of genes involved, it is possible that T1D is a heterogeneous disease depending on the set of genes on which it occurs. At one end of the spectrum, mutations of key immune genes, such AIRE in APECED or FoxP3 in IPEX, are responsible for complex genetic disorders which include autoimmunity T1D. At the other end, environmental factors precipitate diabetes in uncommon forms of T1D, as in the case of congenital rubella in the human or the Kilham virus in the rat. In common forms, it is likely that the respective contribution of susceptibility genes and environmental factors inversely correlates. Environmental factors are possibly as numerous as genes involved, but approaches to screen for environmental factors remain preliminary. Susceptibility variants associate to control quantitative β-cell and immune traits while no unique environmental factor triggers autoimmunity to β-cells, as in the NOD mouse.

**Diabetes autoantigens**

The identification of T1D autoantigens is a key issue [47,48] (table 1). The immune image of β-cells is that of native components of the β-cell membrane in their three dimensional conformation, as seen by B-lymphocytes, and, more importantly, of fragments of intracellular β-cell proteins in the form of 8 to 11mer peptides loaded onto class I MHC molecules, as seen on the β-cell surface by CD8^+ T lymphocytes. In addition, professional APCs present fragments of autoantigens that are phagocytosed following the release of subcellular β-cell particles or debris in the extracellular milieu and loaded onto MHC class I and class II molecules. Given the key role of T lymphocytes in T1D, the cartography of autoantigen-derived peptides that are presented to class I-restricted CD8^+ T-cells and class II-restricted CD4^+ T-cells is of utmost importance. β-cells represent 60 to 70% of the endocrine cells of the islet of Langerhans in which they are surrounded by or interspersed with α, δ and PP cells [49]. The anatomical and physiological organization of the islet of Langerhans is likely to shape the clinical expression of T1D. Factors involved include the local...
### Table 1

**Autoantigens defined as recognized by T-cells in human T1D**

<table>
<thead>
<tr>
<th>Autoantigen</th>
<th>Expression</th>
<th>Subcellular location</th>
<th>Involvement in the NOD mouse</th>
<th>Human T1D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Autoantibodies</td>
<td>CD4+ T-cells</td>
</tr>
<tr>
<td>Insulin</td>
<td>β-cell, thymus</td>
<td>Secretory granule</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAD 65</td>
<td>Neuroendocrine</td>
<td>Synaptic-like microvesicles</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAD 67</td>
<td>Neuroendocrine</td>
<td>Cytosol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IA-2 (ICA512)</td>
<td>Neuroendocrine</td>
<td>Secretory granule</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IA-2 β/phogrin</td>
<td>Neuroendocrine</td>
<td>Secretory granule</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IGRP</td>
<td>β-cell</td>
<td>Endoplasmic reticulum</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Chromogranin</td>
<td>Neuroendocrine</td>
<td>Secretory granule</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ZnT8</td>
<td>β-cell</td>
<td>Secretory granule</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>HSP-60</td>
<td>Ubiquitous</td>
<td>Mitochondria</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>HSP-70</td>
<td></td>
<td></td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>GliMA-38</td>
<td>Secretory granule</td>
<td></td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Amylin/IAPP</td>
<td>Secretory granule</td>
<td></td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>CD38</td>
<td>Ubiquitous</td>
<td></td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

GAD: glutamate decarboxylase; IA-2: islet antigen 2; HSP: heat shock protein; ICA: islet cell antibody. ? no positive results reported. Listing has been limited to autoantigens for which evidence of recognition has been obtained in the human or, if only in the mouse, data are expected in the human.

Homing of infiltrating cells; local presentation of autoantigens; the level of autoantigen expression or expression of neoantigens; and the spreading of the autoimmune reaction from a single to multiple antigenic epitopes (epitope spreading) or from an “initial” autoantigen to other autoantigens. The prediction in the 1980’s that autoimmunity arises against a single autoantigen that is specific of β-cells has not met the experimental evidence. B- and T-lymphocytes recognize several autoantigens in the course of the diabetes process, most of which are co-expressed by β-cells, by other islet endocrine cells and by neurons, in particular glutamic acid decarboxylase (GAD) 65 and 67 and IA-2 (or ICA512 or IA-2β), member of the transmembrane protein tyrosine phosphatase family (table 1). Only insulin and the zinc transporter ZnT8 are specifically expressed by β-cells. The glucose-6-phosphatase catalytic subunit-related protein (IGRP), identified in the NOD, is β-cell-specific, but the evidence that it is involved in human T1D is lacking. All these autoantigens are recognized by autoantibodies that are detected in T1D patients. Anti-IA-2 autoantibodies recognize the IA-2 cytoplasmic domain, which shares 80% sequence homology with another tyrosine phosphatase, phogrin. The striking clustering of autoantigens into intracellular subparticles, i.e. secretory granules and synaptic-like microvesicles, hints at a relationship between key β-cell functions and autoimmune development. IA-2 is located in dense core vesicles of neuroendocrine cells including the β-cells. This, together with the multiplicity of autoantigens, brings indirect evidence that diabetes develops as a defective cross-talk between the islets and immune cells rather than from an intrinsic immune [30,37]. It makes of T1D a β-cell disease rather than an antigen-specific immune disease. The evidence that an autoantigen is critical is obtained by the induction of autoimmunity by immunization against the autoantigen, by transfer of autoantigen-specific T-cell clones, and by the prevention of diabetes upon tolerogenic delivery of the autoantigen in animals that develop the disease spontaneously. Diverse tolerogenic manipulations have been explored, such as the transgenic expression of autoantigens by APCs or β-cells, the use antisense RNA in β-cells to decrease the expression of autoantigens, or by injection of plasmid DNA encoding candidate autoantigens. In addition, the characterization of epitopes recognized by T-cells, such as insulin B9-23, hsp60 p277 or GAD 524-543 has allowed the use of peptides to prevent diabetes in susceptible mice [23,47,48]. The screening of IAβ7-restricted hybridomas obtained by fusing islet-infiltrating T-cells has allowed characterizing proinsulin 1 and 2 epitopes in the NOD [50]. Similar studies in class II−/− mice expressing human class II alleles have allowed characterizing GAD and insulin epitopes restricted by human MHC molecules [51,52].
Among β-cell autoantigens, insulin has been ascribed a key role in the T1D process. In the NOD mouse, injection of insulin-specific T-cell clones accelerates diabetes and diabetes is prevented in prediabetic mice by injecting insulin in incomplete Freund’s adjuvant [53,54]. But the most convincing evidence that an autoantigen is directly involved is by modifying diabetes development in mice in which genes encoding for autoantigens are deleted. Prevention of diabetes and an accelerated form of diabetes are observed in proinsulin 1/− or 2/− NOD mice, respectively, making a strong case for the primary role of insulin in this model [55,56]. Absence of proinsulin 2 expression has been shown to shape the repertoire of insulin-specific T-cells in the periphery and directly influence immune recognition of β-cells [57–59]. By contrast, deficient expression of GAD or IA-2 has no significant effect on diabetes development in this model [60,61]. Antigen spreading may thus explain the activation of T-cells against the long list of autoantigens so far identified once the autoimmune process engaged. T-cell clones that are specific for GAD, chromogranin and IGRP are indeed detected and transfer diabetes into naive NOD recipients [62–65]. In man, insulin and proinsulin are common targets of autoantibodies and T-cells in (pre)diabetic individuals [18,66–72]. Insulin autoantibodies (IAA) are the first to be detected in children at risk for T1D and carry a high positive predictive value for diabetes in siblings of T1D patients [18,66].

**Regulatory T-cell subsets**

Several weeks or months in mouse, or years in man, elapse between initial triggering of the autoimmune response to β-cells and onset of clinical diabetes, defining a prediabetes period during which the detection of insulitis, of autoantibodies or of activated T-cells in the mouse, and of autoantibodies in the human, are detected prior to onset of hyperglycemia. In man, there is even evidence that a high prevalence of autoantibodies is seen as early as by 8 to 10 months of age, with a peak observed between 2 and 3 years, in newborns from a diabetic parent [18,35]. Experimental evidence has been obtained in the NOD and BB rat models that active mechanisms hold back autoreactive T-cells in prediabetes [17,73,74]. Spleen and thymus CD4+ T-cells from young non-diabetic NOD mice prevent the transfer of the disease by splenocytes from diabetic mice [17,74]. In both the NOD mouse and the BB rat, T1D has been associated with a dominant Th1 immune response while Th2 polarization or generation of Treg cells (Th3 and Tr1) has been associated with protection. Regulatory T-cells (Treg) have since been characterized in the mouse as constitutively expressing CD25 (IL-2 receptor α chain). They represent 5–10% of the spleen CD4+ T-cell population, are anergic in vitro and suppress the response of CD4+CD25− T-cells to anti-CD3 activation by inhibiting IL-2 gene transcription and IL-2 production through cognate interaction [75]. Their development requires the presence of the CD28–CD80/CD86 costimulatory pathways. Both CD28− and CD80/CD86−deficient mice NOD mice show profound reduction of the CD4+CD25+ subset and accelerated diabetes [76]. Recent data suggest that TNF-α is implicated in the regulation of the number and function of CD4+CD25+ regulatory T cells (Treg) in NOD mice. The expression of CD62L also allowed defining Treg cell subsets [77,78]. Likewise, Treg cells are found in the CD4+CD25− population in the rat [79]. A breakthrough in the understanding of mechanisms that control the education of T-cells in the thymus has been the demonstration that major tissue-specific autoantigens are expressed in the thymic medullary epithelium under the control of AIRE [80]. Educated Treg cells that express FoxP3 and secrete regulatory cytokines are key cells in controlling autoreactive T-cells in the periphery. Insulin is among the autoantigens that are expressed within the thymic medulla. The level of thymic expression of proinsulin directly impact on the repertoire and function of insulin-specific T-cells in the periphery, as evidenced in proinsulin 2/− NOD mice which show accelerated T1D development [55]. In man, there have been controversial reports documenting a decreased number of CD4+ Treg defined as CD25+ in T1D and few studies of autoantigen-specific Treg which are difficult to detect due to their low frequency and to expand in vitro, since they are characterized by a state of anergy. Both proinflammatory (IFN-γ-producing) and regulatory (IL-10-producing) CD4+ T-cells that are specific of proinsulin and IA-2 epitopes have been characterized in T1D patients and healthy subjects, respectively, and shown suppressive in vitro [81–83]. T1D patients displaying higher IL-10 responses have been characterized by an older age at T1D onset [83]. GAD-specific, DRB1*0401-restricted, CD4+ T-cells have been identified in normal individuals using GAD65-specific class II tetramers (TMs). Their expansion in the presence of peptide was conditioned by the presence of CD4+CD25+ Treg cells [84]. Regulatory CD4+ T-cell that are specific of proinsulin and IA-2 can be cloned in vitro [85,86]. Beyond, CD4+ T-cells, polyclonal regulatory CD8+ T-cells have also been shown to control GAD-specific CD4+ T-cell expansions through a contact dependent mechanism and the production of IL-10 [87].

Other regulatory T-cell subsets play an important role in the control of the autoimmune responses to β-cells. Invariant NKT cells (iNKT) are nonconventional T-lymphocytes restricted by the class I-like CD1d molecule and express an invariant T-cell receptor α chain, Vα14-Jα281 and Vα24-JαQ segments in the mouse and in the human, respectively. They are underrepresented and functionally deficient in IL-4 production in the NOD mouse. The CD1d molecule presents glycolipids. The activation of iNKT cells by synthetic α-galactosylceramide (α-GalCer) ligand prevent diabetes in the NOD mouse. Upon activation, iNKT cells indeed release various cytokines such as IFNγ, IL-4, IL-10, IL-13, TGF-β, IL-3 and GM-CSF and transactivate B-cells,
NK cells and antigen presenting cells. NOD mice that are transgenic for the Vα14-J α281 T-cell receptor have an increased number of NKT cells, an increased production of IL-4 and are protected from diabetes. Cotransfer of transgenic Vα14-J α281 T-cells and diabetogenic splenocytes from non-transgenic diabetic female in SCID recipients confirmed the capacity of NKT cells to prevent diabetes. NKT cell deficiency in CD1<sup>−/−</sup> NOD mice exacerbates diabetes development. The presence in pancreatic lymph nodes of pro-Th2 NKT cells is likely to contribute to their role in preventing diabetes. iNKT cells impair the differentiation of anti-islet T-cells, which become hyporesponsive or anergic [88,89]. Beyond their important role in controlling autoimmune to β-cells, iNKT cells promote immune responses against viruses. In transgenic mice expressing a lymphochoriomeningitis virus (LCMV) protein, iNKT cells have been shown protective, as in the NOD mouse. In this virus-induced model of diabetes, iNKT cells activate plasmacytoid DC (pDC) in the pancreatic islets, enhance the production of type I IFN production and control locally the viral infection [90], inhibiting both antiviral and anti-islet diabetogenic CD8<sup>+</sup> T-cells in pancreatic islets. As reported by many groups, the infection of NOD mice by LCMV exerts a protective effect on diabetes development and the local inhibition of autoimmune effectors by iNKT cells after infection induces an increased frequency of Treg cells that produce TGF-β though their cross-talk with local pDCs, unveiling a new bridge between innate and adaptive immune responses in the prevention of diabetes after viral infection and a new mechanism of protection against diabetes by a contingent infection [91,92].

**Islet-specific T-lymphocytes**

The characterization of epitopes recognized by T-cells in T1D is required for a better understanding of the diabetes process, is a necessary step in the development of T-cell assays in the diagnosis of T1D and prediabetes and is one of the avenues to develop new strategies in immunotherapy of T1D. However, intrinsic difficulties in measuring T-cell responses in the human include the lack of direct access to islet-infiltrating T-cells and the very low frequency of antigen-specific circulating T-cells, the limited sensitivity of technologies such as enzyme-linked immunospot (ELISpot) and flow cytometry to detect these cells. This applies especially to CD4<sup>+</sup> T-cells, due to looser binding constraints in case of class II than class I epitopes.

**T-cell recognition of insulin**

Both direct evidence in the mouse, and indirect evidence in the human, point at insulin as a key autoantigen in T1D autoimmunity. The search for T-cell recognition of insulin and the characterization of insulin epitopes recognized by T-lymphocytes along disease development is thus a major challenge.

**CD4<sup>+</sup> T-cell responses to proinsulin**

CD4<sup>+</sup> T-cell responses to exogenous insulin have first been studied [93]. They are exacerbated in response to inhaled insulin and to some insulin analogs, especially insulin detemir. They are beyond the scope of this review that will only consider T-cell responses to insulin as part of the autoimmune response to β-cells. Autoimmunity to insulin is also seen in the insulin autoimmune syndrome which is responsible for severe hyperglycemia in patients with Grave’s disease treated with antithyroid drugs or patients treated with immunomodulatory drugs such as α-penicillamine [93]. They will not be detailed either. In the NOD mouse, an emphasis has been put on the B chain peptide B9-23 [53,54], but islet-infiltrating T-cells that were specific for a wide array of preproinsulin epitopes were identified without any dominance of any of them [50]. In man, proliferative T-cell responses have been reported against both insulin and proinsulin, especially in recent-onset T1D patients and prediabetic individuals [67,68,94–96], although also in non-diabetic subjects in some reports [71,96]. Given the association of anti-insulin antibodies with HLA-DR4, HLA-DR4-restricted T-cell responses to insulin have first been prioritized. The characterization of the high susceptibility DQ8 molecule led secondarily to the characterization of DQ8-restricted responses. Despite treatment with insulin, long-standing T1D patients were often found low responders [67,95], as also observed in case of CD8<sup>+</sup> T-cells [69]. The possibility that responses to insulin and proinsulin are dissociated has been reported, indicating that some patients respond exclusively to C-peptide, while others respond exclusively to insulin [67]. The possibility that an inverse correlation is seen between the detection of anti-insulin autoantibodies and T-cell responses to proinsulin has been controversial. Epitopes of preproinsulin have been characterized in mice that are transgenic for the human DR1<sup>−/−</sup>0401, DQ8 or DQ6 class II alleles. Immunodominant epitopes were different in the DQ8 mouse and in transgenics that express the DQ6 diabetes-resistant allele [52]. Differences in the epitopes recognized by CD4<sup>+</sup> T-cells from autoantibody-positive individuals at high risk for T1D development and from insulin-treated T1D patients have been reported. Immunodominant epitopes were located within the C-peptide and B chain in the former, within native insulin and insulin B chain in the later [71]. Providing the role of the insulin B chain peptide B9-23 in the NOD mouse and the structural similarities of the murine I-AQ<sup>+</sup> and human DQ8 class II alleles, B9-23 recognition was studied in the human. Short-term T-cell lines obtained from recent-onset T1D patients, while not from controls, were shown highly proliferative to B9-23 and interferon γ-producing cells were detected in recent-onset T1D patients and prediabetic subjects using an ELISpot assay [70], but these data have since not been confirmed in man although presentation of B9-23 was confirmed in DQ8 transgenic mice [97]. Peptides eluted from HLA-DR4 were shown to be
clustered in the C-peptide and C-peptide-A chain region and to elicit interferon γ production exclusively in young T1D patients while IL-10 responses were observed in T1D patients of older age and in controls [83]. Clones obtained from pancreatic draining lymph nodes of two out of three long-standing T1D patients have been shown to recognize predominantly the A chain peptide A1-13, but they were obtained from insulin-treated patients [98]. A DRB1*0401-restricted clone obtained from a prediabetic, autoantibody-positive, child was shown to recognize insulin and the A1-13 peptide providing the presence of a disulfide bond between cysteine residues A6 and A7, showing that posttranslational modifications of insulin epitopes can impact on recognition by autoreactive T-cells [99].

**CD8⁺ T-cell responses to proinsulin**

In contrast with class II molecules that accommodate peptides of very variable length, class I molecules accommodate 8 to 11mers peptides. Several proinsulin epitopes that are presented by HLA class I alleles have been characterized [100]. In case of the common HLA-A*0201 allele, immunogenicity in class I knock out A2.1 transgenic mice has been evidenced [101]. Further studies have characterized proinsulin peptides within the proinsulin B-C region [102] and within the proinsulin leader sequence [103] for recognition by peripheral blood mononuclear cells from A1, A3, A11, A24, B8 and B18 T1D patients. T-cells specific for leader sequence peptide15-24, a peptide that was eluted from HLA-A*0201 molecules, were shown cytotoxic to human islets expressing HLA-A*0201, bringing direct evidence that corresponding T-cells may participate to β-cell destruction in T1D [104]. Most recent-onset T1D patients shows a significant response to at least one of the peptides covering the proinsulin sequence, many showing a response to several peptides, while no response is usually observed in control individuals, including insulin-treated T2D patients. This does not preclude that a more restricted set of peptides is recognized at initiation of the autoimmune process. More surprisingly, proinsulin peptides were recognized both in recent-onset and long-standing diabetic patients [102,105] which may indicate that long term memory class I-restricted T-cells persist in the long term range in patients who have been deprived of residual β cells for years. Long term persistence of memory CD8⁺ T-cells may explain the dramatic recurrence of T1D in recipients of hemigrafts from monozygotic, diabetes-discordant, twins [19]. Recognition of one of the B chain peptides identified, PP33-47/B10-18, was shown to elicit a CD8⁺ T-cell response in long-standing T1D patients who underwent islet graft rejection using an ELISPOT for granzyme, interferon γ and IL-10 production and immunostaining with A2.1-peptide tetramers [106]. The frequency of insulin-specific CD8⁺ T-cells has been estimated at a median frequency of 0.004% (range 0.0008–0.08%) of PMBCs using interferon γ ELISPOT assays [69]. In this last study, they waned within 6 months after diabetes onset. However, as previously mentioned, persistence of CD8⁺ T-cell responses have been observed in long-standing, insulin-treated, T1D patients, in particular to B chain peptides, as opposed to leader sequence peptides. The combination of high sensitivity flow cytometry detection and multiplex fluorescent reagents [107] has been proposed to allow high throughput CD8⁺ T-cell analyses complemented by functional studies in a near future.

**T-cell recognition of GAD, IA-2, IGRP**

Beyond recognition of insulin, T-cell recognition of other autoantigens has been evidenced.

**CD4⁺ T-cells**

Assays and epitopes recognized have been detailed in recent reviews [47,48,100]. Proliferative responses of peripheral mononuclear cells (PMBCs) from T1D patients have initially been reported in the presence of GAD extracts, recombinant GAD and overlapping peptides covering the GAD sequences, but HLA class II restricting molecules have not been defined at that stage and positive responses have also been observed in normal individuals. Difficulties to develop reliable assays in these initial studies may have explained variable outcomes, low reproducibility of data, difficult discrimination of responses between patients and controls and failure to establish standardized assays. DQ-restricted responses to recombinant GAD and proliferative responses have been reported in diabetic or prediabetic individuals against IA-2 or IA-2 peptides have since been defined. The use of transgenic mice expressing HLA class II alleles has helped defining GAD and IA-2 epitopes, leading to identification of candidate DRB1*0401-restricted GAD epitopes [108–110]. Transgenic DQ8 mice have been used to characterize GAD epitopes, including GAD247–266 which shows homology with coxsackie P2-C. DQ8-restricted, GAD599–528-specific, Th1 CD4⁺ T-cell lines were shown to induce insulitis when transferred into DQ8-expressing mice [111]. A strong CD4⁺ T-cell response was observed and human GAD65 epitopes (GAD497–517, GAD527–547, GAD537–557) identified using overlapping peptides spanning two large GAD65 regions in DQ8 transgensics backcrossed onto the NOD background for two generations [112]. The transfer of a DR4-restricted GAD555–567-specific CD4⁺ T-cell clone induces insulitis in Rag1⁻/⁻ B6 DR4 transgensics [113] while CD4⁺ T-cells carrying TCR transgenes from two distinct GAD555–567-specific CD4⁺ T-cell clones in B6 DR4-transgenic mice were shown tolerant [114].

Soluble HLA-DR401 or -DR404 TMs complexed to GAD65 peptides were used to analyze circulating T-cells from recent-onset T1D patients and at-risk subjects have been used to detect high avidity CD4⁺ TMs-positive cells ex vivo or after in vitro expansion and activation on specific plate-bound class II–peptide monomers [115,116]. The generation of HLA-DR*0401-restricted, GAD65-specific, T-cell lines from HLA-DR*0401/DQB1*0302 recent-onset T1D patients has also led
to identification of new DRB1*0401-restricted epitopes while HLA-DR*1601-restricted and HLA-DR*1501-restricted epitopes were also defined [117]. An increase in the avidity of CD4+ T-cell recognition of GAD_{655-667} has been reported in three prediabetic subjects along progression from autoantibody positivity to clinical T1D [118]. Cellular binding assays have allowed identifying clustered epitopes in the GAD C-terminal region and existence of promiscuous peptides binding both diabetes-predisposing and diabetes-protective class II molecules and DR as well as DQ molecules [119–122]. However, limitations in these studies rely with the likelihood that, in contrast with antiviral responses, peptides recognized along the autoimmunity response to β-cells cannot be predicted on an affinity basis [123].

Similar strategies have been used to study CD4+ T-cell recognition of IA-2 [124,125] and IGRP [126], and evidence has been reported for molecular mimicry between an IA-2, as well as GAD, and the rotavirus VP7 protein [127]. Studying a panel of naturally processed islet epitopes by elution from APCs bearing HLA-DR4, IA-2-specific CD4+ T-cells have been identified as proinflammatory T-cells while non-diabetic, HLA-matched controls showed a response against islet peptides, but with an IL-10 secreting phenotype [125].

Autoantigen-specific CD4+ T-cells have been studied in patients undergoing pancreas/kidney or islet transplant [106,128]. Autoantibodies detected pretransplant, or reappearing 5 and 6 years post-transplant in still normoglycemic patients, somewhat paralleled insulitis despite immunosuppression. GAD- and IGRP-specific T-cells detected using TMRs were only temporarily inhibited by immunosuppression.

**CD8+ T-cells**

Following the first evidence for a GAD epitope (GAD_{114-123}) to be the target of cytotoxic, A*0201-restricted, CD8+ T-cells obtained from a subject with preclinical type 1 diabetes and a patient with recent-onset T1D [72], many class I-restricted epitopes derived from GAD, IA-2, IGRP, human islet amyloid polypeptide (IAPP) precursor protein and glial fibrillary acidic protein (GFAP) have now be identified in T1D [47,48,100] following the same approaches as those referred to in case of proproinsulin. Noticeably, IA-2 and GAD are key autoantigens in T1D on the basis of the predictive value of anti-IA2 and anti-GAD autoantibodies in prediabetic individuals. This is not the case for IGRP and IAPP which, however, are defined as targets of CD8+ T-cells in T1D in the human.

**Towards antigen-specific immunotherapy**

While immunotherapy has proved most often efficient in the NOD mouse [129], translation to human T1D has always failed or has faced unfavorable risk/benefit ratios. Immunosuppression has been shown partially effective in preserving β-cells from autoimmune destruction in recent-onset T1D patients at the expense of major side effects that preclude their use in the long-term range [130]. Indeed, conventional insulin therapy has gained in efficacy over years, and a progressively decreasing mortality gap between T1D patients and the general population has been observed [131]. The characterization of autoantigens and peptides recognized by T-cells is thus expected to allow the development of antigen-specific immunotherapy. In focusing treatment on self-reactive T-cells without impairing immune responses to unrelated tumoral or infectious antigens, antigen-specific strategies are expected to avoid risks associated with general immunosuppression.

Immune tolerance is ensured in successive checkpoints by mechanisms affecting differentiating lymphocytes as well as mature lymphocytes in the periphery. Peripheral tolerance relies on mechanisms that are intrinsic to lymphocytes, i.e. deletion of autoreactive lymphocytes, functional ignorance of self-antigens, anergy or immune deviation, and on extrinsic mechanisms, i.e. control of autoreactive lymphocytes by regulatory cells. Molecular interactions involved in the presentation of autoantigens are often central in strategies aiming at restoring or inducing immune tolerance in autoimmunity. Elimination or reprogramming of deleterious autoreactive cells and activation of regulatory cells are major outcomes expected from antigen or peptide-specific immunotherapy. These mechanisms are only starting to be implemented in human studies. It should be underscored that knowing the initiating target autoantigen or autoantigen epitope is not an absolute prerequisite in strategies to induce immune tolerance. Bystander immunomodulation within inflammatory sites is possibly induced through in situ production of protective cytokines, spreading of Th2 responses or deviation toward tolerizing antigen-presentation. This does not preclude advantages in using directly self-antigens. The size of the autoantigen used, its expression pattern, the stage of the disease process at time of immunointervention, the crypticity of the epitopes presented, the autoantigen administration route and dose remain major challenges.

Given the molecular constraints of T-cell activation, the induction of antigen- or peptide-specific tolerance require presentation of antigens or peptides to autoreactive CD4+ T-cells in a non-inflammatory environment as obtained in experimental models by injection of high doses of soluble peptide or antigen, DNA vaccination, the use of the mucosal (oral or nasal) route, intravenous injection of antigen-coupled, ethylene carbodiimide (ECDI)-fixed, splenocytes, or use of altered peptide ligands, either antagonists or partial agonists. Among mechanisms involved, the induction of anergy, at least in part through suboptimal costimulatory signaling, the presentation of the tolerizing epitopes by plasmacytoid dendritic cells, immune deviation or bystander suppression have all been evidenced. However, concerns have been raised by the risk of either exacerbating the autoimmune process or inducing anaphylactic
reactions, as exceptionally reported in multiple sclerosis [132]. Deviation of autoimmunity to different target tissues is another possible concern [133].

**Human trials**

Insulin-based immunotherapy trials have been applied in recent-onset T1D or in autoantibody-positive, prediabetic, subjects. The first trial ever performed used high dose intravenous insulin delivered by an external artificial pancreas in 12 patients to maintain normal glycemic values during 2 weeks at onset of T1D. At one year, C-peptide values were maintained in the intravenous insulin group, while not in 14 NPH insulin-treated patients. Mechanisms may have involved β-cell rest or immunomodulation through intravenous delivery of insulin [134]. By contrast, in a larger group of patients, the DCCT study did not find any difference between intensive insulin therapy, although not intravenous insulin treatment, and a conventional insulin treatment [135].

A randomized, double-blind, crossover study in 38 autoantibody-positive, prediabetic individuals showed that nasal insulin delivery has immune effects, including an increase of anti-insulin antibodies and a decrease in proliferative T-cell responses to insulin. No acceleration of diabetes was observed and the first phase insulin response to glucose was stabilized in 26 individuals who did not developed diabetes at one year [136]. The Diabetes Prevention Trial-Type 1 Diabetes Study [137] assigned 339 autoantibody-positive individuals with a projected 5-year risk over 50% to observation or low-dose subcutaneous ultralente insulin, 0.25 U/kg/d in addition to annual 4 days courses of continuous intravenous insulin infusions and showed no significant difference between the two groups. In subjects with a five year projected risk of 26 to 50%, assignment to oral insulin versus placebo made no difference either. However, in a subgroup with high anti-insulin autoantibodies levels, 6.2% individuals receiving oral insulin developed T1D, as compared to 10.4% in the placebo group (P < 0.015) [138]. A more recent, double-blind, randomised study in 115 subjects with ≥ 2 autoantibodies under intranasal insulin (1 U/kg/d) showed no difference with a control group [139]. Seemingly, oral administration of insulin or insulin B-chain in incomplete Freund’s adjuvant failed to show any benefit on C-peptide at one year [140–142]. The use of early sulfonylurea treatment versus insulin in anti-GAD positive patients with a 5-year non-insulin-dependent diabetes profile has shown controversial results [143,144]. The most recent trials that have used GAD in GAD autoantibody-positive T1D patients [145,146] or the p277 peptide derived from Heat shock protein 60 (hsp60) have not confirmed the initial indications that a benefit could be obtained on residual insulin secretion [147].

**Conclusion**

Preventing T1D with the goal of restoring or inducing immune tolerance to β-cells is a major challenge in human T1D. It precludes development of T-cell assays to diagnose diabetes autoimmunity. New T-cell technologies are expected to allow defining autoreactive T-cell differentiation programs and characterizing autoimmune responses in comparison to physiological immune responses. This may allow additional mechanistic studies and prove instrumental in the discovery of immune correlates of efficacy in clinical trials [148,149]. The ultimate challenge is to get better insight into the natural history of diabetes in its very early stages and define the initial environmental trigger of autoimmunity to β-cells, if any, or the initial dysregulation in the physiological cross-talk between immune cells and β-cells that leads to T1D.

**Disclosure of interest:** the author declares that he has no conflicts of interest concerning this article.

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