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Thymic self-antigens for the design of a negative/tolerogenic self-vaccination against type 1 diabetes

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Before being able to react against infectious nonself-antigens, the immune system has to be educated in the recognition and tolerance of neuroendocrine proteins and this critical process takes place only in the thymus. The development of the autoimmune diabetogenic response results from a thymus dysfunction in programing central self-tolerance to pancreatic insulin-secreting islet β cells, leading to the breakdown of immune homeostasis with an enrichment of islet β-cell reactive effector T cells and a deficiency of β-cell specific natural regulatory T cells (nTregs) in the peripheral T-lymphocyte repertoire. Insulin-like growth factor 2 (IGF-2) is the dominant member of the insulin family expressed during fetal life by the thymic epithelium under the control of the autoimmune regulator (AIRE) gene/protein. The very low degree of insulin gene transcription in normal murine and human thymus explains why the insulin protein is poorly tolerogenic as demonstrated in many studies, including the failure of all clinical trials that have attempted immune tolerance to islet β cells via various methods of insulin administration. On the basis of the close homology and crosstolerance between insulin, the primary T1D autoantigen, and IGF-2, the dominant self-antigen of the insulin family, a novel type of vaccination, so-called 'negative/tolerogenic self-vaccination', is currently being developed for the prevention and cure of T1D. If this approach were found to be effective for reprograming immunological tolerance in T1D, it could pave the way for the design of other self-vaccines against autoimmune endocrine diseases, as well as other organ-specific autoimmune diseases.

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"Autoimmune disease can be a depressing subject. In Shakespearian terms, 'it is a tale told by an idiot…signifying nothing'. In more modern metaphor, it is an error made at random in an enormous, delicately programmed computer. Nature has no other way of handling genetic error than by eliminating the faulty, and the physician handling autoimmune diseases can expect no help from her."

Sir F. MacFarlane Burnet, 1972

Introduction

In 1965, our late Belgian colleague Willy Gepts observed inflammatory infiltrates of mononuclear cells invading Langerhans' islets in the pancreas of deceased young diabetic patients [1]. In a prophetical analysis, he discussed his innovative results with the following words: *'It seems probable that, in the pancreas of acute diabetics, we had the opportunity to catch the final stages of a process which has been going on for an indefinite time, perhaps from birth on'*. Since this pioneering work, research conducted worldwide has firmly established that type 1 diabetes (T1D) — previously called juvenile diabetes, and insulin-dependent diabetes — is the final result of a highly selective autoimmune response that generates an inflammation (insulitis), followed by the death of insulin-secreting islet β cells in the pancreas. Incidence of T1D peaks around 10–14 years and this disease affects ±20 million people worldwide (approximately 10% of all patients with diabetes mellitus). The mean prevalence of T1D in Europe is about 8 new cases per year and per 100 000 individuals, but this prevalence is five to six times higher in Scandinavian countries, particularly in Finland.

Humoral and cellular immune responses of T1D

The discovery of autoantibodies directed against Langerhans' islet cells was a crucial step for further demonstrating the autoimmune nature of the pathogenic process in T1D [2]. Since then, the nature of autoantigens targeted by these autoantibodies has been well defined, and the three major T1D-related autoantigens are (pro)insulin, the 65-kDa isoform of glutamic acid decarboxylase (GAD65), and the tyrosine phosphatase IA-2. The islet-specific cation efflux transporter ZnT8 (Slc30A8) and chromogranin A were also recently reported as important autoantigens in T1D [3,4]. However, from these autoantigens, only antigenic epitopes derived from (pro)insulin are specific of pancreatic islet β cells. Furthermore, there is now ample evidence that autoimmunity to (pro)insulin is central to autoimmune diabetes pathogenesis both in nonobese diabetic (NOD) mice and in humans [5,6]. Anti-insulin, anti-GAD65, and anti-IA-2 autoantibodies are very reliable markers of the autoimmune response targeting β cells. Serum from more than 90% of children with recent-onset T1D contains antibodies against one or several of these autoantigens. Their high predictive value is also established since they can be detected several years before the clinical signs of insulin deficiency. The predictive value of autoantibodies against several autoantigens is higher than high titers of one single autoantibody. If the three autoantibodies are detected in one individual, the risk of developing T1D is at least 80 times higher than in the general population. The combination of autoantibodies with susceptible genetic alleles of the major histocompatibility (MHC) class II locus further increases this predictive value. Such prediction is very useful for clinical studies targeted at T1D prevention given the relatively low incidence of this disease [7,8]. However, the pathogenic significance of T1D-related autoantibodies is rather low, if not absent [9], and the principal effectors of β-cell autoimmune destruction are CD4+ and CD8+ T lymphocytes [10]. Investigation of specific T-cell responses in T1D patients is very difficult because of the low frequency in the peripheral T-cell pool of autoreactive T cells specific of epitopes derived from (pro)insulin, GAD65 or IA-2. However, the development of sensitive and specific techniques, such as enzyme-linked immunosorbant spot assays (ELISpot) and tetramers of class I/II HLA molecules complexed with T1D-related epitopes, has already provided very significant data that further document the importance of T-cell mediated mechanisms in T1D pathogenesis [11,12].

Genetic factors in T1D pathogenesis

T1D is the polygenic autoimmune disease that has been most intensively investigated at the genetic level. Knowledge of genetic loci that determine susceptibility to T1D is important for identifying pathogenic pathways, for improved prediction of the disease, and for selection of potential pharmacological targets. The balance between susceptibility and resistance alleles determines individual predisposition to T1D. The most significant part (±50%) of genetic susceptibility to T1D resides in the HLA class II region on chromosome 6p21, as recognized by pioneering studies [13,14]. The major susceptibility in this region is conferred by the specific HLA class II haplotypes DR4-*DQA1*0301-DQB1*0302* (DQ8 molecule) and DR3-*DQA1*0501-DQB1*0201* (DQ2 molecule). In contrast, the allele *DQB1*0602* (DQ6 molecule) confers dominant protection against T1D. Theoretically, HLA class I proteins present antigens that are processed from endogenous proteins to CD8+ T cells, while HLA class II proteins present antigens issued from exogenous proteins to CD4+ T cells. Consequently, it has long been difficult to explain the relationship between insulin and T1D genetic susceptibility located in the HLA class II region. This problem was solved when very elegant crystallographic studies showed that a dominant insulin epitope (Ins B9–23) is presented in the binding pocket of DQ8 and DQ2 proteins [15••]. Since then, a comprehensive scan of the whole HLA region, combined with potent statistical methods, has also linked T1D susceptibility to HLA class I genes *HLA-B* and *HLA-A* [16].

Other genetic linkage and association studies have identified a second locus for T1D susceptibility that corresponds to a high polymorphic mini-satellite constituted by a variable number of tandem repeats (VNTR) [17,18]. This VNTR is embedded on chromosome 11p15, and controls the transcription of the insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) genes downstream. Short VNTR class I alleles contain 20–63 repeats of 14–15 base pairs, while intermediate class II and long class III alleles include 64–139 and 140–210 repeats, respectively. VNTR class I alleles are associated with TID susceptibility, whereas class III alleles confer protection.

The *CTLA4* gene region on chromosome 2q33 is also associated with susceptibility to T1D [19]. The signaling between B7, expressed by professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed by T cells, plays a pivotal role in peripheral T-cell tolerance. CTLA-4 is expressed neither by thymocytes (thymic T cells) nor by resting T cells, but it is detectable after antigen-mediated T-cell activation, and downregulates responses of activated T cells. *Ctla4* deletion in mice results in an extremely severe lymphoproliferative and an autoimmune phenotype with lethal multiorgan tissue destruction [20•].

Another mutation in a non-HLA gene conferring significant susceptibility to T1D is a variant of the lymphoid tyrosine phosphatase Lyp gene (*PTPN22*), a suppressor of T-cell activation [21•]. Lyp normally interacts with a C-terminal Src kinase (Csk) complex to dephosphorylate

positive regulatory tyrosines and downregulate signaling from the T-cell receptor (TCR) pathway. The minor allele derived from the single-nucleotide polymorphism (SNP) differs in a single but crucial amino acid residue (R620W) involved in the interaction of Lyp with Csk. Interestingly, the same variant R620W also increases risk to other common autoimmune diseases, such as rheumatoid arthritis, Graves' disease, and systemic lupus erythematosus [22]. However, the variant *PTPN22* 620W is a gain-of-function mutant, since it is associated with a higher catalytic activity of the encoded Lyp, a marked decrease of T-cell response to antigen stimulation, CD25 expression, and IL-10 secretion from TCR stimulation, and an increase in peripheral memory CD4+ T cells [23,24]. The role of this mutation in the pathogenesis of T1D and other autoimmune diseases remains to be further elucidated.

Different studies, including a genome-wide association analysis, have identified association of T1D with noncoding SNPs on the chromosome 10p15 region containing CD25, which encodes the high-affinity α chain of the IL2R complex [25•]. Further mapping of the association between the *IL2RA* locus and T1D supported a role of IL2Rα in the pathogenesis of the disease, most possibly through modulation of regulatory T-cell (Treg) activity [26].

An association has also been found between T1D and a polymorphism of the IGF2 receptor gene (*IGF2R*), which seems to be subject to parental imprinting since only maternal alleles at this polymorphism are associated with the disease [27]. Human T1D differs from other common autoimmune disorders, which preferentially affect females (e.g. autoimmune diabetes in the NOD mouse). Evidence was also recently provided for an association between T1D and polymorphisms in *CYP27B1*, which encodes 1α-hydroxylase, the enzyme that transforms 25(OH) vitamin D into bioactive $1,25(OH)_2$ vitamin D3 [28].

Environmental factors

Many observations strongly support an important influence of environmental factors in the pathogenesis of T1D: the lack of complete concordance in monozygotic twins (approximately 30% of them develop T1D), the fact that less than 10% of genetically susceptible individuals progress to overt disease, the increase in T1D annual incidence observed in recent years, as well as the higher incidence of new cases between March and October. Geographic localization also determines important variation in T1D incidence when one compares high-rated Northern European countries such as Finland (40–45 new cases/100 000 inhabitants per year) and low-rated countries such as Venezuela and China (0.1/100 000 inhabitants per year) [29]. Migrant populations moving from low-incidence countries tend to acquire the same risk as the inhabitants of the welcoming countries. For example, T1D annual incidence among Pakistani children living in United Kingdom is identical to that observed among English children (e.g. 11.7/100 000 inhabitants versus 1/100 000 inhabitants in Pakistan) [30]. However, migrant studies also provide evidence for the importance of genetic background, since the risk of T1D remains increased for people migrating from high-incidence areas to low-risk countries [31]. In Europe, a 10-fold North–South gradient is observed in T1D incidence (with the noticeable exception of Sardinia for unknown reasons). Owing to the relative homogeneity of European populations, such a gradient cannot result from genetic differences only. Although childhood T1D was rare and lethal at the beginning of the 20th century, a recent European Community Concerted Action Program (EURODIAB) has shown that new T1D cases in European children under 5 years is predicted to double between 2005 and 2020, and prevalence of T1D cases under 15 years will rise by 70% [32]. As discussed by the authors, these rapid changes over relatively short periods of time cannot be explained by changes in prevalence of susceptibility genes. Among environmental influences, several studies have pointed to modern lifestyle habits, increased weight and height, increased caesarean deliveries and, most perhaps importantly, the 'hygiene hypothesis'. This hypothesis proposes that the decrease of childhood infections and other environmental stimuli impair the healthy development and diversification of the neonatal immune repertoire, inducing higher incidence of allergic and autoimmune diseases later in life [33•,34•]. In the NOD mouse, a classic animal model of T1D, the susceptibility to autoimmune diabetes is also greatly affected by environmental effects, and the incidence of the disease is much higher when NOD mice are bred in a germ-free environment [35].

A number of viral infections have also been associated with the subsequent development of T1D including enteroviruses, congenital rubella, mumps, cytomegalovirus, Epstein–Barr virus, and varicella zoster virus [36,37]. Epidemiological studies have provided the strongest evidence that coxsackievirus (CVB) and other enterovirus infections are frequent events in subjects who ultimately develop T1D [38]. CVB4 is the most common serotype detected in prediabetic and diabetic individuals. The CVB4 strain E2 has been isolated from the pancreas of an acutely deceased diabetic child, passed through murine islet β cells, and then found to induce a diabetes-like disease after inoculation in mice [39]. Early epidemiologic studies have suggested that CVB may be involved in T1D pathogenesis. Using serological analyses, initial first studies showed that newly diagnosed T1D patients were more frequently positive for CVB4 than control subjects [40,41]. Subsequently, a series of epidemiological studies have confirmed high frequencies of IgM anti-CVB in children recently diagnosed with

T1D. Thereafter, using RT-PCR detection of the virus genome, Clements *et al.* showed that 64% of children at onset of T1D were positive for enteroviruses as opposed to 4% of controls [42]. In another study, CVB genome was detected in five out of 12 (42%) newly diagnosed T1D patients and in one of 12 (8%) patients during the course of the disease. By contrast, none of 12 T2D patients and none of 15 healthy adults had enterovirus sequences in their blood [43]. The CVB4 strain E2 is able to induce a persistent infection of human islet β cells [44], whereas a new isolated CVB4 variant, VD2921, causes a persistent infection of islet β cells with a consequent disturbance of proinsulin synthesis and insulin secretion [45]. CVB4 E2 and VD2921 genomes were recently detected by RT-PCR in the peripheral blood mononuclear cells (PBMCs) of a majority of T1D children at the onset of their diabetes. The presence of enterovirus RNA in the blood cells of most new T1D children supports the hypothesis that a viral infection is involved in T1D pathogenesis. Interestingly, six out of seven controls positive for CVB4 had been infected by a phylogenetic branch of CVB4 different from the one detected in diabetic patients, suggesting the existence of CVB4-related substrains with different diabetogenic effects [46].

Despite a significant homology between the amino acid sequence 28–50 of P2-C, a nonstructural viral protein of the CVB4 replicative complex, and amino acids 250–273 of the β-cell autoantigen GAD65, molecular mimicry is not involved in CVB-induced diabetes, as mice with susceptible MHC alleles do not show CVB-induced acceleration of diabetes [47]. Moreover, none of anti-GAD65 antibodies produced by lymphocytes isolated from a newly diagnosed T1D patient crossreacted with the protein P2-C itself [48]. Nevertheless, it was shown that a viral epitope mimicking a β-cell antigen is able to accelerate, but not to prime a diabetogenic autoimmune process [49]. A very recent study has also identified a molecular mimicry between human T-cell epitopes in rotavirus and pancreatic islet autoantigens (GAD65 and IA-2) [50].

An alternative potential mechanism is a CVB-mediated 'bystander' activation of autoreactive T cells against islet antigens; this mechanism was proposed to explain the rapid onset of diabetes in mice carrying a TCR specific for a sequestered islet autoantigen. In that model, CVB induces diabetes by a direct local infection, leading to inflammation, secondary tissue damage, and then release of sequestered islet antigens that are able to stimulate resting autoreactive T cells [47]. According to those observations, autoreactive T lymphocytes would gain access to the target islets without being involved in the initial viral insult or in reactivity to the viral antigens [51]. The same group also provided strong evidence that the early innate immune response to CVB4 is responsible for β-cell damage and the development of diabetes. Indeed, β cells became highly susceptible to CVB4 infection and subsequent NK cell response after inhibition of interferon (IFN) signaling by transgenic overexpression in islet β cells of suppressor of cytokine signaling 1 (SOCS-1) under the influence of the insulin promoter. The islet β cells were secondarily damaged by apoptosis occurring during the innate immune response, rather than by the adaptive B-cell and T-cell responses. Thus, target β cell defense critically influences susceptibility to T1D after CVB4 infection [52].

Although the relationships between CVB infection and subsequent T1D development are still debated by some authors, recent studies using PCR techniques with very specific oligonucleotide probes — thus avoiding serological pitfalls and crossreactions — have found substantial evidence for an association between a previous CVB infection and T1D. High levels of IFN-α, an indirect indicator of viral infection, were measured in 70% of 56 new type 1 diabetics, together with positive detection of CVB RNA in ±50% of the IFN-α positive patients [53]. Somewhat ironically, the association between T1D and viral infections has been recently reinforced by genetic studies that have shown a linkage between T1D susceptibility and host genetic determinants of the antiviral responses such as the antiviral oligoadenylate synthetase (*OAS1*) and the interferon-induced helicase (*IFIH1* or *MDA5*), which intervenes in innate immunity by recognition of RNA genomes of picornaviruses (such as coxsakieviruses) [54–56]. Therefore, the question of a higher incidence of enterovirus infection during childhood in countries with a high risk of T1D deserves to be further investigated, particularly if one seriously considers the possibility of anti-CVB vaccination as a potential method for T1D prevention in these areas.

The central role of the thymus in self-tolerance of neuroendocrine proteins and the nature of 'neuroendocrine self'

A major question when addressing the pathogenesis of organ-specific autoimmunity such as T1D is the origin of the self-reactive T cells that are directed against target antigens of endocrine cells. Among all lymphoid structures, the thymus is an organ that emerged some 500 million years ago, concomitantly or very shortly after recombinase-dependent adaptive immunity, with a specific function of orchestrating central immunological self-tolerance. The thymus is not an endocrine gland, but it crucially stands at the intersection between the immune and neuroendocrine systems. In this organ that is responsible for thymopoiesis, that is, generation of naïve and competent T lymphocytes, the neuroendocrine system regulates the process of T-cell differentiation from very early stages, while in parallel naïve T lymphocytes are educated to recognize and tolerate neuroendocrine gene/protein families [57,58•,59]. Therefore, the thymus is a unique organ where a constant conflict occurs

between ancient, highly conserved, neuroendocrine proteins and a more recently evolved system equipped with recombination machinery for promoting stochastic generation of T-cell response diversity. Contrary to popular opinion, the thymus continues to function throughout life and plays a fundamental role in the recovery of a competent T-cell repertoire after intensive chemotherapy or during highly active antiretroviral chemotherapy in human immunodeficiency virus infection [60,61]. The integrity of the somatotrope growth hormone/IGF-1 axis is known to be important for the maintenance of thymus function in adult life [62•].

The thymus constitutes the central arm of immunological self-tolerance by two essential mechanisms that are intimately associated with, and paradoxically mediated by, the same thymic self-antigens: first, clonal deletion of self-reactive T cells issued from the random recombination of TCR genes (negative selection) and second, generation of self-antigen-specific natural regulatory T cells (nTregs) that are able to inactivate in periphery self-reactive T cells having escaped intrathymic negative selection [63••,64].

For a long time, peripheral tissue-restricted antigens targeted by autoimmune processes were thought to be sequestered from T cells during their intrathymic differentiation. We and several other groups have demonstrated that thymic epithelial cells (TECs) from different species constitute a site for the promiscuous transcription of a great number of genes encoding tissue-restricted antigens or belonging to neuroendocrine families, such as the neurohypophysial family, tachykinins, neurotensins, somatostatins, atrial natriuretic peptides, and the insulin family. This demonstration has radically changed our common understanding of the pathogenesis of organ-specific autoimmune endocrine diseases such as T1D. From the investigation of intrathymic expression of neuroendocrine-related self-peptide precursor genes, a series of properties can be derived that define the nature of the 'neuroendocrine self'. First, thymic neuroendocrine self-antigens usually correspond to peptide sequences that have been highly conserved throughout the evolution of their related family. Second, a hierarchy characterizes their expression pattern in the thymus. In the neurohypophysial family, oxytocin (OT) is the dominant peptide synthesized by TECs from different species. The binding of OT to its cognate receptor expressed by pre-T cells induces a very rapid phosphorylation of focal adhesion related kinases. This event could play a major role in promoting establishment of synapses between immature T lymphocytes and thymic APCs, TECs, macrophages, and DCs. With regard to tachykinins, neurokinin A (NKA) — but not substance P — is the peptide generated from the processing by TEC of the preprotachykinin A (*PPT-A*) gene product. All the genes of the insulin family are expressed in the thymus according to a precise hierarchy and topography during fetal life: *IGF2* (cortical and medullary TECs) > *IGF1* (thymic macrophages) ≫ *INS* (a few subsets of medullary TECs). This hierarchical pattern is meaningful because the strength of self-tolerance to a protein is proportional to its intrathymic concentration [65]. Third, neuroendocrine precursors are not processed according to the classic model of neurosecretion, but they undergo an antigenic processing for presentation by — or in association with — MHC proteins [66••]. Fourth, most of neuroendocrine self-antigens are transcribed in the thymic epithelium under the control of the autoimmune regulator gene *AIRE* (see below). Fifth, intrathymic *OT* transcription precedes OT and vasopressin (VP) expression in hypothalamic magnocellular neurons. Finally, epigenetic regulation of intrathymic gene expression is strongly suggested by the loss of *IGF2* parental imprinting and overexpression in human medullary TECs [67,68].

This hierarchy in the organization of the thymic repertoire of neuroendocrine self-antigens is also significant from an evolutionary point of view. Since a series of essential and physiological functions had been established before the appearance of adaptive immunity in cartilaginous fishes, they had to be protected from the risk of autotoxicity inherent to this type of immunity. For example, OT is a 'bonding' peptide that has been implicated at different steps of the reproductive process, and thus for species preservation possibly had to be protected to a greater degree than VP, which controls water metabolism and vascular tone. Along the same line of reasoning, IGF-2 as a major factor in fetal development possibly had to be more protected than insulin, which is 'only' responsible for glucose homeostasis. Nevertheless, because of their close homology, thymic neuroendocrine self-antigens may promote crosstolerance to other members of their respective families. This was recently demonstrated by the weaker tolerance to insulin of $Igf2^{-/-}$ mice when compared to wild-type mice [69•]. Further insight into the discrimination between the relative influence of central and peripheral arms of immunological self-tolerance will be gained through the generation of mice with TEC-specific *Igf2* deletion, currently under development in our laboratory.

The central role of a thymus dysfunction in T1D pathogenesis (Figure 1)

As hypothesized by Burnet in 1973, the essential pathogenesis of autoimmune diseases may first depend on the appearance of 'forbidden' self-reactive clones in the peripheral T-cell repertoire [70]. In 1992, a defect in the process of intrathymic T-cell education to recognize and to tolerate OT was hypothesized to play a pivotal role in the development of hypothalamus-specific autoimmunity leading to 'idiopathic' central diabetes insipidus [71]. The progressive increase in the degree of immune diversity and complexity may explain why failures in self-

tolerance are increasingly detected during evolution with most such failures occurring in the human species. Since the thymus is the primary site for induction of self-tolerance, thorough investigation of the mechanisms responsible for a breakdown of thymus-dependent tolerance should provide the scientific community with important keys to understand the mechanisms underlying the development of autoimmune responses. This was the principal objective of the European FP6 Integrated Project Euro-Thymaide. A number of abnormalities of thymic morphology and cytoarchitecture have been described for several autoimmune disorders. Central tolerance and apoptosis of self-reactive T cells are defective in the thymus of NOD mouse [72,73]. Transcription of insulin-related genes (*Ins*, *Igf1*, and *Igf2*) has been analyzed in the thymus of diabetes-resistant (BBDR) and diabetes-prone (BBDP) rats, another model of T1D. *Ins* and *Igf1* transcripts were detected in all thymi from BBDP and BBDR rats. *Igf2* transcripts were also present in the thymus from all BBDR rats, but were not detected in the thymus from more than 80% of BBDP rats, in close concordance with the incidence (86%) of autoimmune diabetes in those rats. This defect in *Igf2* transcription in BBDP thymus could contribute to both their lymphopenia (including CD8+ T cells and suppressor/regulatory RT6+ T cells) and to the absence of central self-tolerance to insulin-secreting islet β cells [74,75]. Other authors have shown that susceptibility to autoimmune diabetes is correlated with the level of *Ins2* transcription in the mouse thymus [76]. Breeding of $Ins2^{-/-}$ mice onto the NOD background markedly accelerated insulitis and onset of diabetes [77]. In contrast, insulitis and diabetes were considerably reduced in $Ins1^{-/-}$ congenic NOD mice [78]. These observations are explained by the dominance of *Ins2* encoding proinsulin in the murine thymus, while *Ins1* encodes proinsulin in islet β cells. In the human species, *INS* transcripts were measured at lower levels in the fetal thymus with short class I VNTR (variable number of tandem repeats) alleles, a genetic trait of T1D susceptibility as discussed above [79•,80•]. The fundamental role of thymic insulin in mediating central self-tolerance of islet β cells was definitively demonstrated by the rapid onset of autoimmune diabetes following thymus-specific deletion of *Ins1* and *Ins2* through an elegant transgenic construction in mice [81••].

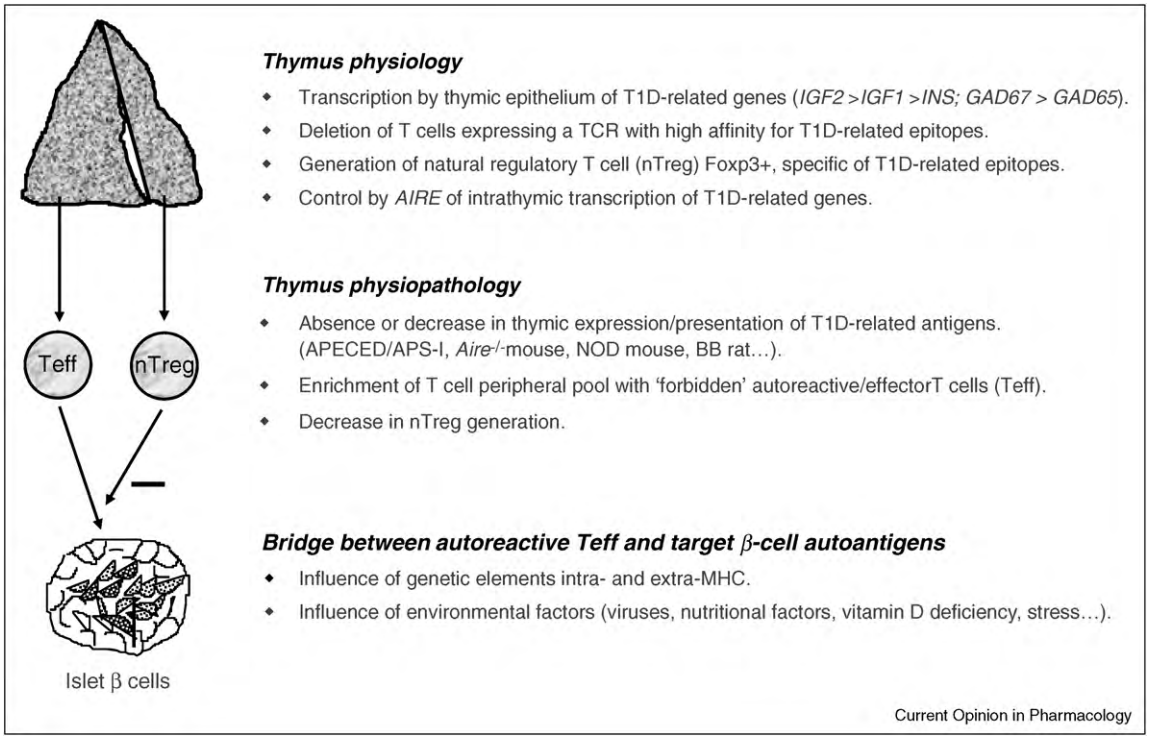
The identification of *AIRE* led to further demonstration that a thymus dysfunction plays a crucial role in the pathogenesis of organ-specific autoimmune diseases [82,83]. Loss-of-function *AIRE* single mutations are responsible for a very rare autosomal recessive disease named autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy (APECED), or autoimmune polyendocrine syndrome type 1 (APS-1). This syndrome develops in early childhood and is characterized by multiorgan autoimmunity and insufficiency of several endocrine glands such as parathyroids, adrenal cortex, and gonads. *AIRE* codes for a 54-kDa protein sharing structural characteristics with transcription factors. Its expression is maximal in the thymus, mainly in medullary TECs, but is absent in TECs of NOD mice [84]. Depending on their genetic background, $Aire^{-/-}$ mice exhibit several signs of peripheral autoimmunity, which are associated with a significant decrease in thymic transcription of neuroendocrine genes (including *Ot*, *Npy*, *Igf2*, and *Ins2*), as well as other tissue-specific genes [85••,86••,87••]. Of note, *Aire* deficiency on NOD background induces both wasting and resistance to diabetes, while autoimmunity severely affects pancreatic exocrine acini [88].

In collaboration with Didier Hober (Laboratory of Virology EA3610, CHRU Lille, France), we have shown that CVB4 is capable to directly infect the epithelial and lymphoid compartments of the human and murine thymus, and to induce a severe thymus dysfunction with massive pre-T-cell depletion and marked upregulation of MHC class I expression by TECs and by CD4+ CD8+ immature thymic T cells [89,90]. Interestingly, outbred mice can be infected with CVB4 following an oral inoculation, which results in systemic spreading of viral RNA and a prolonged detection of CVB4 RNA in thymus, spleen, and blood up to 70 days postinoculation [91]. These findings suggest that thymic CVB4-mediated severe infection could enhance CVB4 virulence through induction of immunological tolerance to CVB4, and consequently may play a role in the breakdown of central self-tolerance to islet β cells.

Self-vaccination as an alternative for T1D prevention and cure (Figure 2)

Given the impossibility to modify the genetic constitution of susceptible individuals and to act efficiently upon most of the environmental influences — except perhaps through a future anti-enterovirus/CVB4 vaccination in high-risk countries — contemporary clinical results still favor an immunomodulatory approach aiming to control the autoimmune response oriented against β cells, but without compromising general immunity. Ideally, this autoimmune regulation should be combined with strategies of regeneration of damaged islet β cells and the inhibition of the apoptotic process promoted in β cells by the autoimmune process. Nevertheless, even after transplantation of β cells from allogenic or xenogenic donors, or β cells issued from adequate differentiation of embryonic stem cells or induced pluripotent stem cells, the control of autoimmune memory selective of islet β cells is an absolute prerequisite both for T1D prevention and for cure. Until now, significant clinical success has been reached only with Fc receptor (FcR)-nonbinding CD3-specific humanized monoclonal antibodies that were shown to preserve endogenous insulin-secreting islet β-cell mass in recently diagnosed T1D patients

Figure 1

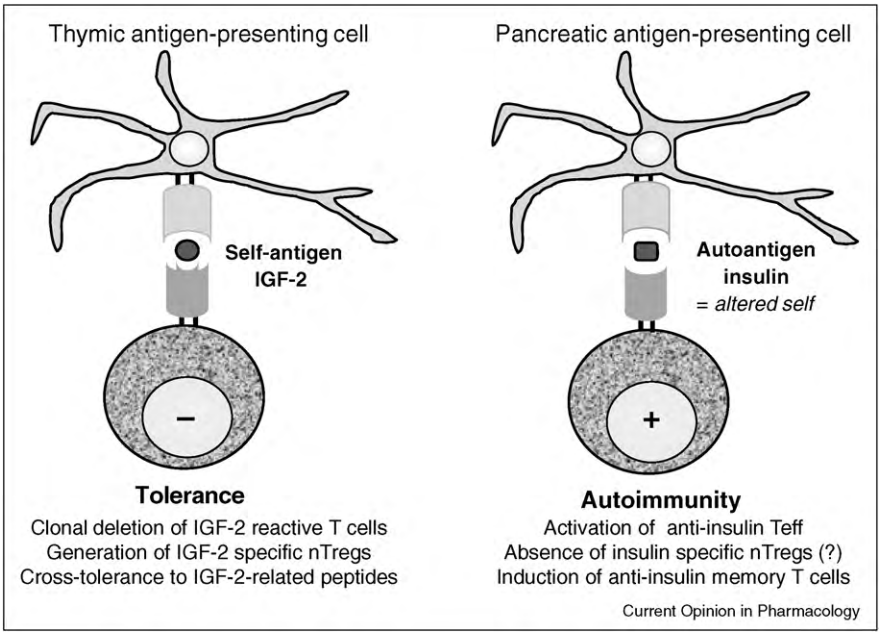


Thymus physiopathology and T1D development. Throughout life, the thymus selects Teff self-tolerant and competent against nonself-antigens, and generates self-specific nTregs. Thymic epithelium transcribes genes encoding T1D-related antigens, as well as other neuroendocrine-related and tissue-restricted antigens, under *AIRE* control for most of them. Absence or decrease in the presentation of thymic T1D-related antigens (as observed in different animal models of autoimmune diabetes) conducts to the enrichment of the peripheral T-cell pool with 'forbidden' self-reactive T cells bearing TCR directed against T1D-related epitopes, while thymic generation of specific nTregs is severely impaired. Combination of these two events is responsible for the breakdown of central self-tolerance to islet β cells. Both genetic and environmental factors are involved in the establishment of a molecular bridge between anti-β cell autoreactive Teff and islet β-cell autoantigens. Once this bridge is formed, the autoimmune pathogenic response is triggered and leads to a progressive reduction of the β-cell mass.

[92•,93•]. This strategy of immunomodulation in T1D has been extensively discussed elsewhere [94].

Because of its antigen-specificity, the most attractive immunomodulating approach is the design of peptide-based therapeutic vaccines [95–97]. A recent randomized, placebo-controlled clinical trial has shown that two subcutaneous injections of GAD65 (20 μg) in a standard vaccine formulation with alum (GAD-alum) contribute to the preservation of residual insulin secretion in recent-onset T1D, but did not change the insulin requirement [98,99]. According to the novel knowledge gained in T1D pathogenesis and the central role of a thymus dysfunction in its development, the control of the autoimmune process could be obtained by (re)programing β-cell through the potent tolerogenic properties of the thymus, in particular the repertoire of thymic T1D-related self-antigens. According to this perspective, the profile of cytokine secretion was analyzed after presentation of Ins B9–23, a major T1D autoantigen [5,6], and the homologous sequence IGF-2 B11–25 derived from IGF-2, the dominant thymic self-antigen of the insulin family. This study was performed in PBMC cultures derived from DQ8-positive T1 adolescents. First, InsB9-23 and IGF-2 B11-25 were shown to have the same affinity and to compete for binding to DQ8 and DQ2 (Wücherpfennig and Geenen, unpublished data). Second, using ELISpot methodology, DQ8 presentation of IGF-2 B11 25 was found to induce a regulatory profile (↑IL-10, ↑IL-10/IFN-γ, and ↑IL-4), statistically different from the profile induced by Ins B9–23. This regulatory profile could derive from a different cytokine profile secreted by Ins B9–23-reactive CD4+ T cells in response to IGF-2 B11–25, or from the recruitment and activation of IGF-2 specific Tregs. So, contrary to insulin, the 'altered self-IGF-2', IGF-2 and derived epitopes might be a much more appropriate choice for a novel type of a negative self-vaccination that associates competition for MHC presentation and regulatory responses downstream, as well as potential bystander suppression of autoimmune responses to other T1D-related autoantigens. This hypothesis is currently being investigated by vaccination of NOD mice with recombinant human IGF-2 alone or in combination with adjuvants. A very recent study has shown that the combination

Figure 2



Principles of negative/tolerogenic self-vaccination. These principles are based on homology and crosstolerance between IGF-2 and insulin. Intrathymic presentation of IGF-2 as the self-antigen of the insulin family leads to clonal deletion of IGF-2 reactive T cells and generation of IGF-2 specific nTregs. The diabetogenic autoimmune response results from recognition of insulin (as 'altered IGF-2') and activation of anti-insulin Teff having escaped thymic censorship. It could also be facilitated by the unproved absence of insulin-specific nTregs. IGF-2 antigenic epitopes compete with homologous insulin sequences for binding to MHC, and their recognition by anti-insulin TCRs might promote a regulatory response (↑IL-10, ↑IL-4) instead of an inflammatory Th1 profile.

of antigen-based therapy with FcR-nonbinding CD3-specific monoclonal antibody strongly increased the activity of insulin-specific Foxp3+ CD4+ CD25+ Tregs. These cells could transfer dominant tolerance to immunocompetent recent-onset diabetic mice recipients, and they were shown to secrete IL-10, TGF-β, and IL-4, thus strongly suggesting induction of antigen-specific Tregs [100]. Finally, with regard to generation of islet β cells from human induced pluripotent stem cells, IGF-2 was used with nicotinamide for the final differentiation of pancreatic exocrine/endocrine cells into insulin-producing cells [101]. It is notable that these results suggest that the same protein, IGF-2, can be employed both to regenerate the functional β-cell mass and to reprogram of immunological tolerance to islet β cells.

Conclusion

The thymus plays a central role in the establishment of central immunological self-tolerance toward Langerhans' insulin-secreting islet β cells, and there is now evidence that the development of T1D results from a breakdown of thymus-dependent tolerance of insulin-family derived epitopes. This knowledge should translate in the very near future to the design of novel tolerogenic/regulatory approaches aimed at restoring the immunological tolerance specific of islet β cells, which represents an appealing strategy for both the prevention and the cure of T1D, one of the heaviest prices paid by the human species for having evolved the advantage of the extreme diversity and efficiency of adaptive immune responses against new biological threats.

Conflict of interest statement

VG is coinventor of IGF-2 related patents. No other conflict of interest relevant to this article was reported.

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